METHIMAZOLE ADMINISTRATION TO CATS:

IN VIVO AND *IN VITRO* STUDIES

OF TRANSDERMAL ABSORPTION

A thesis presented in partial fulfilment
of the requirements for the degree of

Doctor or Philosophy
in
Veterinary Science

at Massey University, Manawatu
New Zealand.

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2015

Dedication:

To Mum and Dad,

Thank-you for instilling the love of education and science,

Thank-you for instilling the love of life,

Thank- you for your love and patience,

Mum, I miss you each day.

Dad, I love you and love our Skype chats.

ABSTRACT

The use of medications formulated as ointments or gels and applied to the inner pinna of cats has become popular in veterinary medicine due to the ease of administration by this route compared to oral administration. Benign hyperplasia of the thyroid is a very common condition in cats older than ten years of age. Medical therapy with anti-thyroid drugs such as methimazole or the pro-drug of methimazole, carbimazole, is one of the treatment options for cats with hyperthyroidism. All previous studies of methimazole applied to the inner ear of cats have used Pluronic® lecithin organogel as the vehicle, however carbimazole and methimazole are lipophilic drugs, and PLO gel might not be the most suitable vehicle for a lipid soluble drug.

A series of studies were designed to test a new, lipophilic formulation of carbimazole and subsequently a new formulation of methimazole for transdermal application to the inner ear of cats for the therapy of hyperthyroidism. Two pharmacokinetic studies in healthy cats, one pilot trial and one clinical trial in client owned hyperthyroid cats, established that the lipophilic formulation could be absorbed from the ear and was a safe and efficacious therapy for hyperthyroidism in cats. A drug company (Bomac Ltd, now Bayer NZ Ltd) was interested in the novel formulation and the product was patented (International Application Number PCT/NZ2008/000011). The commercial product containing the drug and vehicle was sold in New Zealand as Hyper-T™ Earspot. Finally, a series of three *in vitro* studies were performed to determine that methimazole in the lipophilic vehicle was: a) absorbed across the pinnal skin; b) absorbed more completely at that site than the same formulation applied to the neck, groin or thoracic skin; and c) able to penetrate from the inner to the outer ear of cats in an *in vitro* model.

These studies represent the most extensive studies to date of a drug applied to the inner pinna of cats. The results from these studies suggest that methimazole in a lipophilic vehicle can be absorbed across the skin of cats and is an efficacious therapy for the treatment of hyperthyroidism in cats.

ACKNOWLEDGEMENTS

could be here to read this. To Annabel, who provided so much support in these closing stages of the PhD, I owe a lifetime supply of Belgian chocolate. To my immediate family, my husband Fred, for his patience and understanding and who has been there as the major emotional and physical support throughout this rollercoaster ride. To our children, Else and Klara, who have been born during this process, we look forward to our new adventures together, where mum is not always working on her PhD. To number three, we are all looking forward to meeting you, however please wait until this is submitted. Addendum: to Beatrix, thank you for waiting 16 hours after submission to arrive into this world and bring us joy.

LIST OF PUBLICATIONS

Nanjan, K., Chambers, P., Hill, K. E., & Al Alawi, F. (2007, December 20). *552816, Topical formulation*. Intellectual Property of New Zealand.

    International Application Number PCT/NZ2008/000011

    US Patent number US 8,748,467 B2

Hill, K. E., Gieseg, M. A., Kingsbury, D., Lopez-Villalobos, N., Bridges, J., & Chambers, J.P. (2009): The efficacy and safety of a novel lipophilic formulation methimazole for the once daily transdermal treatment of cats with hyperthyroidism. *Abstract of the 2009 ACVIM Forum/Canadian VMA convention*. *Journal of Veterinary Internal Medicine*, *23*, 756.

Hill, K. E., Gieseg, M. A., Kingsbury, D., Lopez-Villalobos, N., Bridges, J., & Chambers, J.P. (2011): The Efficacy and Safety of a Novel Lipophilic Formulation of Methimazole for the Once Daily Transdermal Treatment of Cats with Hyperthyroidism. *Journal of Veterinary Internal Medicine*, *25*(6), 1357-1365.doi: 10.1111/j.1939-1676.2011.00799.x

Hill, K.E, Gieseg, M.A., Bridges, J., & Chambers, J.P. (2014): The pharmacokinetics of methimazole in a novel lipophilic formulation administered transdermally to healthy cats. *New Zealand veterinary journal*, *62*(4), 208-213. doi: 10.1080/00480169.2013.875990

Hill, K. E., Mills, P. C., Jones, B. R., Bolwell, C. F., Aberdein, D., & Chambers., J. P: (2015): Percutaneous absorption of methimazole: an in vitro study of the absorption pharmacokinetics for two different vehicles. *Journal of Veterinary Pharmacology and Therapeutics*. doi: 10.1111/jvp.12213.

Hill, K. E., Chambers., J. P, Jones, B. R., Bolwell, C. F., Aberdein, D., & Mills, P. C. (2015a): Regional variations in percutaneous absorption of methimazole: an *in vitro* study on cat skin. *Journal of Veterinary Pharmacology and Therapeutics*. doi: 10.1111/jvp.12220.

Hill K.E., Chambers J.P, Jones B.R, Bolwell C.F., Aberdein D, & Mills P.C. (2015b): Trans-pinnal movement of methimazole: an *in vitro* study showing that methimazole can cross from the inner to outer pinna of cats. *Journal of Feline Medicine and Surgery*. doi: 10.1177/1098612X14567548.

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Chapter 4: THE EFFICACY AND SAFETY OF A NOVEL LIPOPHILIC FORMULATION OF METHIMAZOLE FOR THE ONCE DAILY TRANSDERMAL TREATMENT OF CATS WITH HYPERTHYROIDISM

Hill, KE, Gieseg, MA, Kingsbury DD, Bridges J, Lopez-Villalobos, Chambers JP: The efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism. *Journal of Veterinary Internal Medicine*. 25:1357–1365, 2011.

Chapter 5: PERCUTANEOUS ABSORPTION OF METHIMAZOLE: AN *IN VITRO* STUDY OF THE ABSORPTION PHARMACOKINETICS FOR TWO DIFFERENT VEHICLES

Hill K.E., Mills P.C., Jones B.R, Bolwell C.F., Aberdein D, Chambers J.P: Percutaneous absorption of methimazole: an *in vitro* study of the absorption pharmacokinetics for two different vehicles. *Journal of Veterinary Pharmacology and Therapeutics*. doi: 10.1111/jvp.12213; 2015

Chapter 6: REGIONAL VARIATIONS IN PERCUTANEOUS ABSORPTION OF METHIMAZOLE: AN *IN VITRO* STUDY ON CAT SKIN

Hill K.E., Chambers J.P, Jones B.R, Bolwell C.F., Aberdein D, Mills P.C.: Regional variations in percutaneous absorption of methimazole: an *in vitro* study on cat skin. *Journal of Veterinary Pharmacology and Therapeutics*. doi: 10.1111/jvp.12220; 2015

Chapter 7: TRANS-PINNAL MOVEMENT OF METHIMAZOLE: AN *IN VITRO* STUDY SHOWING THAT METHIMAZOLE CAN CROSS FROM THE INNER TO OUTER PINNA OF CATS

Hill K.E., Chambers J.P, Jones B.R, Bolwell C.F., Aberdein D, Mills P.C: Trans-pinnal movement of methimazole: an *in vitro* study showing that methimazole can cross from the inner to outer pinna of cats. *Journal of Feline Medicine and Surgery*. doi: 10.1177/1098612X14567548; 2015

Appendix D: STATEMENTS OF CONTRIBUTION TO DOCTORAL
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LIST OF ABBREVIATIONS

^{131}I	Radioactive iodine	fT4	free T4
ALP	Alkaline phosphatase	h	Hour
ALT	Alanine transaminase	HPLC	High performance liquid chromatography
ANA	Antinuclear antibodies	I^-	Iodide
AST	Aspartate transaminase	INN	International Nonproprietary Name
AUC	Area under the curve	LOD	Limit of detection
BID	Twice a day	LOQ	Limit of quantification
BSA	Bovine Serum Albumin	Ltd	Limited
CBC	Complete blood cell count	MHC	Major Histocompatibility
CBZ	Carbimazole	MIT	Mono-iodotyrosine
CI	Confidence interval	MMI	Methimazole
C_{max}	Maximum concentration	Na^+	Sodium
DIT	Diiodotyrosine	NZ	New Zealand
FDA	Federal Drug Agency	OECD	Organisation for Economic Co-operation and Development

PBS	Phosphate buffered saline	TBG	Thyroid binding globulin
PCAA	Professional Compounding Association of America	TBPA	Thyroxine binding pre-albumin
PLO	Pluronic® lecithin organogel	TD	Transdermal
PO	per os	t_{max}	Time at maximum concentration
PTU	Propylthiouracil	TRH	Thyroid releasing hormone
rT3	reverse T3	TSH	Thyroid-stimulating hormone
SD	Standard deviation	TT4	Total thyroxine concentrations
SID	Once a day	UA	Urinalysis
$t_{½}$	Elimination half-life	USA	United States of America
T3	Tri-iodothyronine	USAN	United States Adopted Name
T4	Thyroxine	USG	Urine-specific gravity

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Phosphate buffered saline with 4% bovine serum albumin. Means with different superscripts within row are different (p < 0.05).

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wavelength was 252 nm, run time 15 minutes. Methimazole standards were run before all samples were analyzed, the 40 ng Methimazole (MMI) standard with the peak shown between 7 and 8 minutes is illustrated below.

Chapter 4

Chapter 5

discs of two older intact male cats and four young cats using an *in vitro* Franz cell model. The error bars represent 95% confidence intervals.

Chapter 6

Figure 1: Cumulative methimazole (MMI) with vertical error bars representing 95% confidence intervals in six cats after application of 10 mg of methimazole to skin of the ear, neck, groin or thorax over 36 hours. Each data point represents the geometric mean of two replicates from six cats. The cumulative amount of methimazole per µg/mL is on the y axis. There was a significant difference ($p < 0.001$) of region.

Figure 2: Mean methimazole absorption (flux) (MMI) with vertical error bars representing 95% confidence intervals in six cats after application of 10 mg of methimazole to skin of the ear, neck, groin or thorax using a concentrations over 36 hours. Each data point represents the geometric mean of two replicates from six cats. There was a significant difference ($p < 0.001$) of region.

Figure 3: Haematoxylin and eosin (H&E) (1000x) stained images of the groin (A), neck (B), thorax (C) and left ear inner epidermis (D) of cats in the study on the effect of region on the absorption of transdermal methimazole. The stratum corneum layer (SC) and epidermal (E) layers are labelled in B. A significant difference in region was found for the epidermis ($p < 0.001$). The epidermis was thinner for the groin compared to the ear ($p = 0.001$) and the ear and thorax ($p = 0.006$). There was no difference in skin region for the thickness of the stratum corneum ($p = 0.601$).

Chapter 7

Figure 1: Two chambered Franz type diffusion cell used in the study on percutaneous absorption of methimazole in the cat. The skin is clamped between the upper donor compartment and lower receptor compartment. The receptor compartment solution simulates the physical conditions surrounding the subcutaneous tissues. All cells were mounted in a diffusion apparatus, and placed in a water bath set to maintain the temperature of the skin in the diffusion cell at approximately 32°C. The receptor compartment solution is magnetically stirred. Samples of the receptor solution are removed via the sampling port at set time points, with equal volumes of fresh solution being replaced into the port.

Figure 2: In an *in vitro* study showing that methimazole can cross from the inner to outer pinna of cats, one cat had a neuter tattoo that penetrated the cartilage. After application of 10 mg of methimazole to the inner pinna and the whole ear placed in an *in vitro* Franz cell for 30 h, the right ear of this cat had 3.3 mg of methimazole in the cartilage, the highest of all the cats in the study.
A = Cartilage, B= inner ear.

Figure 3: The mean amount (mg/g) of methimazole recovered from six cat whole ear samples (11 ears) after application of 10 mg of methimazole in a lipophilic vehicle to the inner pinna and the whole ear placed in an *in vitro*

Franz cell for 30 h, stratified by site (the right ear of one cat was excluded due to abnormally high concentrations of methimazole). The y-axis shows the amount of methimazole (mg/g), the standard deviation is shown with bars. After adjusting for the effect of ear (left or right), no significant difference was found in methimazole concentration between the different sites of the ear (inner ear, cartilage, outer ear) (p=0.47). A difference was found between the total methimazole concentration between the left and right ear *p < 0.001).

Appendix A

Appendix B

averaged. (Monteiro-Riviere *et al*. 1990). An example of three measured regions of stratum corneum (cornified, non viable epidermis) (SC 1–3) and viable epidermis (E 1–3), are shown in this figure.

Appendix C

Introduction

THESIS STRUCTURE

The studies presented in this thesis are in the form of manuscripts published in peer reviewed journals, and are formatted for the style of the journal they were published in. Consequently there is some repetition of background information and methods in some of the Chapters, and also the units (SI or common) depends on the style of the journal. All manuscripts have been standardised to one referencing style throughout the thesis. Extra information that may have been removed from each Chapter before publication, is included as an appendix at the conclusion of each Chapter. References are included at the end of each Chapter.

Throughout **Chapters 2-7**, figures and tables are labelled as Figure 1, Table 1 etc, in line with the published manuscript. As such, there are multiple Figure 1s etc throughout the thesis, however where required, each figure is clearly identified as **Chapter 2, Figure 1** etc. At the end of the thesis, further information on the materials and methods of the *in vitro* studies and further exploration of the data obtained in the *in vitro* studies is included as **Appendix A** and **B**. **Appendix A** expands on some of the methods of the *in vitro* Franz type cell studies, as well as combining data from **Chapters 5** and **6** to explore for any trends in the data. The appendix is included after **Chapters 5** and **6**, so that the reader may understand why some of this data exploration was performed. **Appendix B** describes the skin histology performed on the skin samples from the studies in **Chapters 5-7**. Data has been combined for the ear histology, providing an overview of the inner pinnal histology from 18 cats, where as in **Chapters 5-7**, only 6 cats are discussed. Again

this chapter was included as an Appendix after **Chapters 5-7** in the hope that the reader is more familiar with the data, and can also refer to the Appendix concurrently when reading **Chapters 5-7**. Further information on the methods of high performance liquid chromatography (HPLC) analysis of methimazole is found in **Appendix C**.

The science of transdermal drug therapy is a rapidly expanding field in both veterinary and human medicine. The body of literature and the scientific language used to describe aspects in this scientific field is expanding. Throughout the thesis, terms such as transdermal carbimazole/methimazole and transdermally (adverb) are used to be fully descriptive and to reduce words and length of sentences without losing accuracy of description. These terms can be found in the literature and medical dictionaries (such as http://medicine.academic.ru/94276/transdermal), as the language surrounding this field develops.

THESIS OUTLINE

Chapter 1 of the thesis is a literature review, which provides the reader a brief overview of hyperthyroidism in cats, then moves on to the therapy of hyperthyroidism, particularly medical therapy with drugs such as carbimazole and methimazole. An overview of the use of the transdermal application of drugs for therapeutic use in cats is covered, as well as a brief overview of how drugs can be studied using *in vitro* models. The literature review reveals that there are many questions still unanswered in regards to the use of drugs for transdermal application in cats.

The aim of the pilot trial described in **Chapter 2** is to test the theory that carbimazole, the drug used to treat hyperthyroidism in cats in New Zealand, if formulated into a lipophilic vehicle for transdermal application to the inner pinna of healthy cats, will be absorbed for systemic action. A description of the formulation is provided and a discussion on the methods developed to measure serum methimazole concentrations in cats. From this study, funding was obtained from a New Zealand drug company (Bomac Ltd) to further explore the use of this drug for the therapy of hyperthyroidism in cats, and preliminary patents were obtained for the drug in this novel vehicle. **Chapter 2** was not a peer reviewed published manuscript.

The manuscript in **Chapter 3** describes the pharmacokinetics of methimazole in a novel lipophilic vehicle in healthy cats. This study further refines the methods for measuring methimazole concentrations in the serum of cats and compares the novel formulation of methimazole with methimazole in Pluronic® lecithin organogel (PLO) and carbimazole tablets *per os*.

Chapter 4 reports the results of the clinical trial in client owned hyperthyroid cats of the comparison of oral carbimazole, with the novel lipophilic formulation of transdermal methimazole applied to the pinna once a day.

Chapter 5 is the first of three *in vitro* studies using the Franz type cell model and harvested feline skin. A description of the percutaneous absorption pharmacokinetics of methimazole in a novel lipophilic vehicle compared to the application of methimazole in PLO gel on feline ears using a finite dose in an *in vitro* Franz cell model is provided. There is discussion on the variability of absorption found between cats and also discussion on the histology of the pinnal skin.

Chapter 6 is the second *in vitro* study. The aim of the study was to compare the *in vitro* absorption of methimazole in the novel lipophilic formulation through feline skin collected from different regions of the body. The outcomes from this *in vitro* study were to determine if methimazole in a lipophilic vehicle is likely to penetrate feline skin, and therefore be systemically active, when applied to different body regions. An important outcome of the study was to describe if the pinna is the most suitable site to apply this topical formulation of methimazole in the cat.

Chapter 7 completes the trio of *in vitro* studies. The aim of this study is to determine if the percutaneous application of a methimazole in a lipophilic vehicle, applied to the internal pinna (non-haired region) will cross to the external (haired) pinna of the ear in an *in vitro* Franz type cell model.

To conclude, in the final chapter of this thesis, a discussion of the key findings of this research is provided, including the limitations and recommendations for further research.

Appendix A and **B** provide more information on the general methods of the in vitro studies, some of which was removed from **Chapters 5-7** when the manuscripts were published. Some of the data from **Chapters 5-7** is also combined, and explored to look for any trends and a brief discussion of this data is provided. The reader is encouraged to refer to **Appendix A** and **B** (concurrently) while reading **Chapters 5-7**.

Chapter 1

Literature review

1.0 OVERVIEW OF HYPERTHYROIDISM IN CATS

1.1 Introduction

Hyperthyroidism or thyrotoxicosis, is a disorder which results from the hyper secretion of thyroid hormones from the thyroid gland resulting in excessive concentrations of thyroid hormones (thyroxine [T4] and or tri-iodothyronine [T3]) in circulation. Feline hyperthyroidism was first discovered in the USA in the late 1970s (Holzworth et al., 1980; Peterson & Ward, 2007) and was first reported in NZ by Jones and Johnstone in 1981 (Jones & Johnstone, 1981). Since the discovery of hyperthyroidism, the incidence of disease has increased from 1 in 1000 cats seen by veterinarians in 1978-1982 to 21 in 1000 from 1993-1997 of all cats examined by veterinarians (Edinboro et al., 2004).

The criteria for the diagnosis of hyperthyroidism in cats are similar to that in humans. Criteria include an elevated serum thyroxine concentration and clinical signs of weight loss, polyphagia, polyuria and tachycardia, along with a palpable thyroid nodule (Peterson et al., 1983).

Hyperthyroidism has been documented throughout most of the world (Peterson, 2012). A recent, large study of cats examined in primary practice in England, established an apparent prevalence of 2.4% (95% CI 2.3 to 2.5 per cent) in all cats, and 8.7% (95% CI 8.3 to 9.0 per cent) in cats aged 10 years or above (Stephens et al., 2014). Data

published in 2014 by a large chain of primary practice hospitals in the USA (Banfield Pet Hospital® group) showed that around 7% of cats over the age of 10 years of age where diagnosed with hyperthyroidism (Anonymous, 2014). A Japanese study reported the prevalence in cats older than 9 years of age in the Osaka and Chugoku regions was 8.9% (Miyamoto et al., 2002), in Germany cats older than 8 years of age had a reported an incidence of 11.4% (Sassnau, 2006). In the most recent survey in Warsaw, Poland 20.1% in cats aged seven years and older were hyperthyroid (Gójska-Zygner et al., 2014). The lowest prevalence in cats older than 10 years was documented in Hong Kong (4%) (De Wet et al., 2009). Hyperthyroidism is now recognised as the most commonly diagnosed endocrinopathy in small animal practice (Mooney, 2010).

1.2 Thyroid anatomy, physiology and embryology

The thyroid is located caudal to the larynx, on either side of the trachea. It is a small bi-lobed gland, and in the cat, is only occasionally connected by an isthmus of thyroid tissue (Figure 1.1.1) (Dyce et al., 2010).
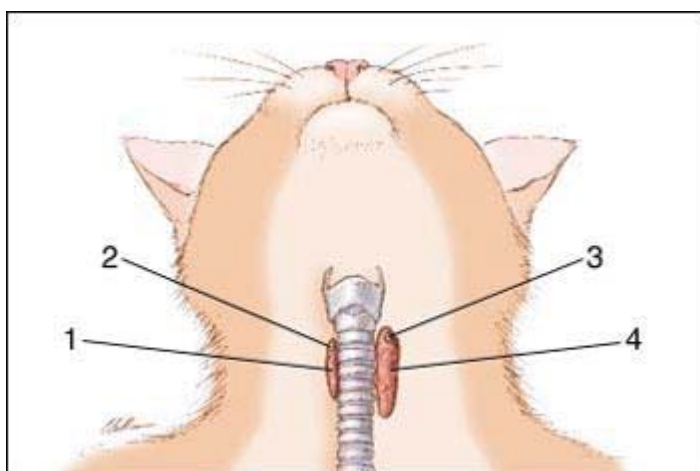
Figure 1.1.1: The thyroid gland is located on each side of a cat's trachea. The gland on the left (1) is normal sized, the gland on the right (4) is enlarged. Numbers 2 and 3 are the parathyroid glands. Image from: https://castlevetsreading.files.wordpress.com/2014/10/fthyroid-1.jpg

The thyroid gland consists of two functional cell types – the follicular cells and the para-follicular cells. Follicular cells produce thyroid hormones tri-iodothyronine (T3) and thyroxine (T4), and para-follicular cells produce calcitonin. The thyroid gland is composed of large numbers of follicles, filled with colloid (Figure 1.1.2) which is composed of the large glycoprotein thyroglobulin.
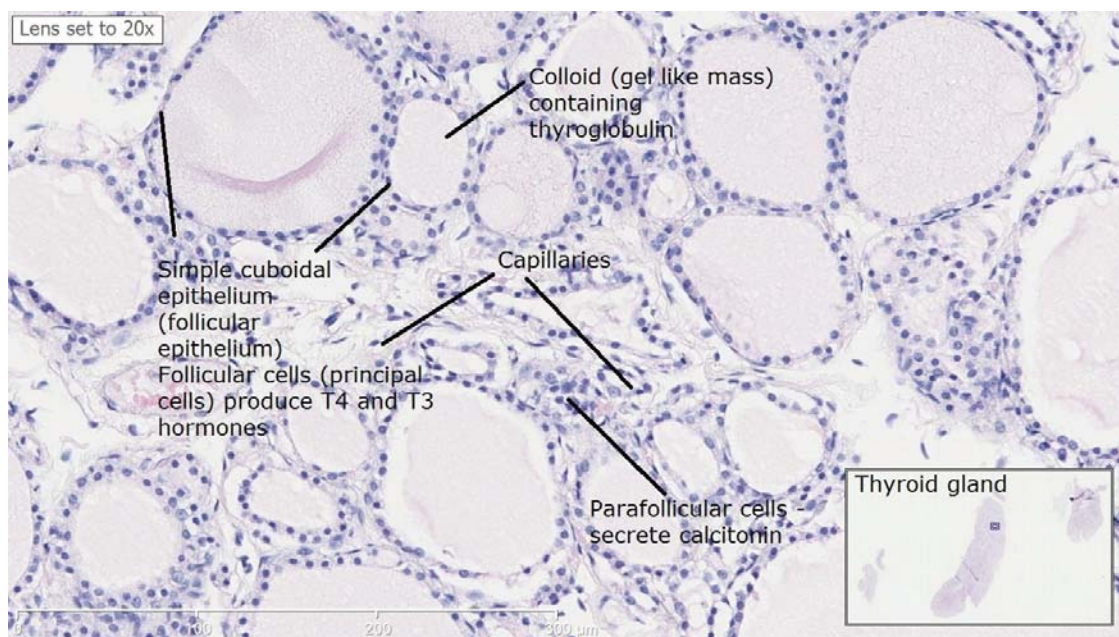
Figure 1.1.2: The thyroid gland is composed of many follicles that are filled with colloid. Bar = 300 µm. Image from :
http://www.onlineveterinaryanatomy.net/sites/default/files/original_media/image/asset_9190_41%20-%20thyroid%20gland%20labelled%20feline.jpg

Thyroid hormones are synthesized within the follicle cell (Figure 1.1.3) in the following steps.

1. Iodine that is ingested is converted to iodide and absorbed; and iodide is taken up by the thyroid gland. The thyroid gland concentrates iodide (I⁻) by active transport against a concentration gradient using the sodium – iodide transporter (iodide pump) The accumulation of iodide is dependent on the active transport of sodium (Na^+) that couples the energy released by the inward translocation of Na^+ down to its electrochemical gradient to the simultaneous inward translocation of I⁻ against its electrochemical gradient.

2. Thyroglobulin is synthesized in the thyroid cells and secreted into the colloid. Iodide is rapidly oxidized by thyroid peroxidase and bound to tyrosine residues in thyroglobulin.

3. Thyroxine (T4) and tri-iodothyronine (T3) are synthesized in the colloid by iodination (3) and condensation (4) of tyrosine molecules bound to thyroglobulin. Iodinated tyrosine residues mono-iodotyrosine (MIT) and, diiodotyrosine (DIT) in thyroglobulin combine to form iodothyronines (T3 and T4). The hormones are bound to thyroglobulin until secreted.

5. When T4 and T3 are secreted, thyroglobulin in the colloid is ingested by endocytosis by the follicular cells, the peptide bonds are hydrolyzed and free T4 and T3 are discharged into the capillaries. Some of the T4 is converted to T3 and some released as T4 into the blood stream. Some of the T3 is also formed in peripheral tissues by deiodination (T4). Most idotyrosines (MIT and DIT) are deiodinated and iodine is recycled. T4 is preferentially secreted by the thyroid gland, with only small amounts of T3 and reverse T3 (rT3) secreted.

6. In the blood stream 99% of T4 and T3 are bound to plasma proteins (Thyroid binding globulin (TBG), thyroxine binding pre-albumin (TBPA), albumin). Only the <u>free form</u> of the hormone is able to enter the cell membrane, binding to receptors to be biologically active. The protein bound hormone acts to maintain a plasma reservoir and a steady state of the free hormone. Free T4 is mono-iodinated within the cell to deiodinated T3 or rT3 depending on the metabolic demands at the time. In normal metabolic states, T3 is preferentially produced, but in illness, starvation or catabolic metabolism, production of rT3 is increased and production of T3 decreased. Intracellular T3 binds to receptors within the cell and produces the main physiological response. Circulating T4 is thought to be responsible for the feedback control of the secretion of TSH and TRH from the pituitary and the hypothalamus. Twenty percent (20%) of circulating T3 is derived from the thyroid gland; the rest is from peripheral deiodination of T4 to T3.

7. T3 and T4 are metabolized in the liver and kidneys and many other tissues. In the liver T4 and T3 are conjugated and excreted in the bile.
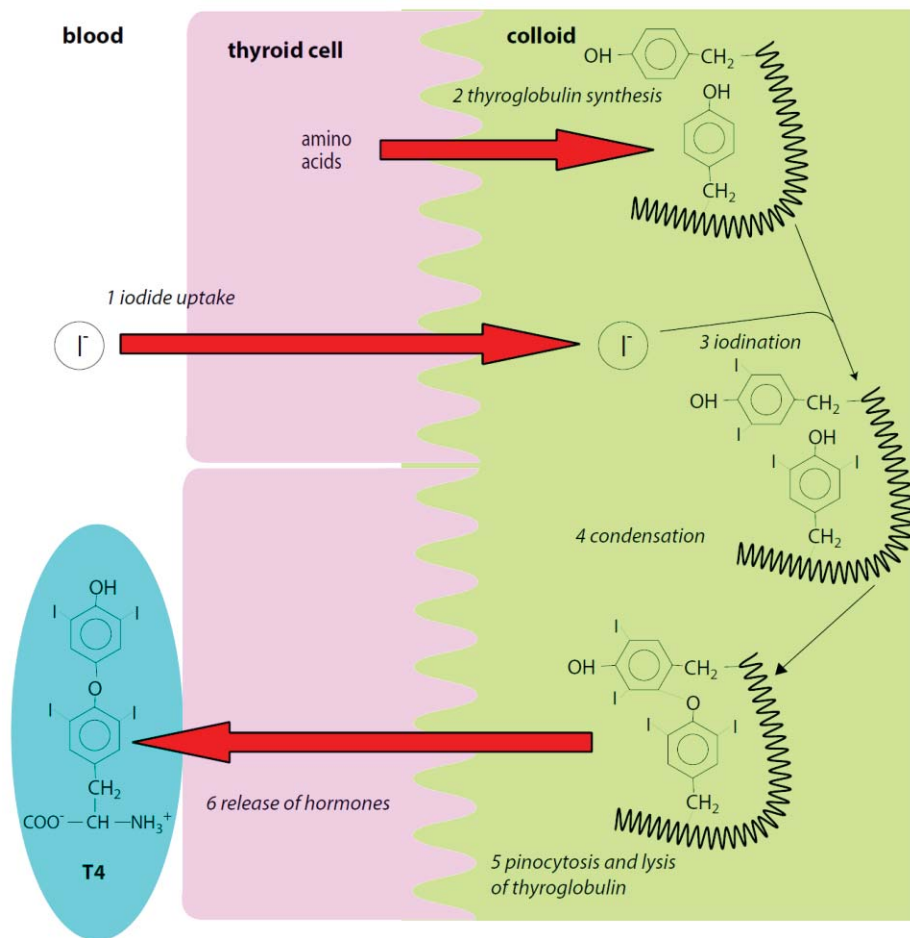
Figure 1.1.3: The synthesis of thyroid hormones within the follicle cell. 1) Iodine that is ingested is converted to iodide and absorbed; and iodide is taken up by the thyroid gland. 2) Thyroglobulin is synthesized in the thyroid cells and secreted into the colloid. Thyroxine (T4) and tri-iodothyronine (T3) are synthesized in the colloid by iodination (3) and condensation (4) of tyrosine molecules bound to thyroglobulin. 5) thyroglobulin in the colloid is ingested by endocytosis by the follicular cells. 6) Release of T4 and T3 to the blood stream, T4 is preferentially secreted by the thyroid gland, with only small amounts of T3 and reverse T3 (rT3) secreted.

Thyroid secretion is controlled by thyroid-stimulating hormone (TSH) secreted by the anterior pituitary (Figure 1.1.4). TSH binds to the TSH receptor on the thyroid follicular cells and increases the production and secretion T_3 and T_4. The secretion of TSH from the pituitary is under control of the Thyroid releasing hormone (TRH) which is produced in the medial neurons of the paraventricular nucleus of the hypothalamus.
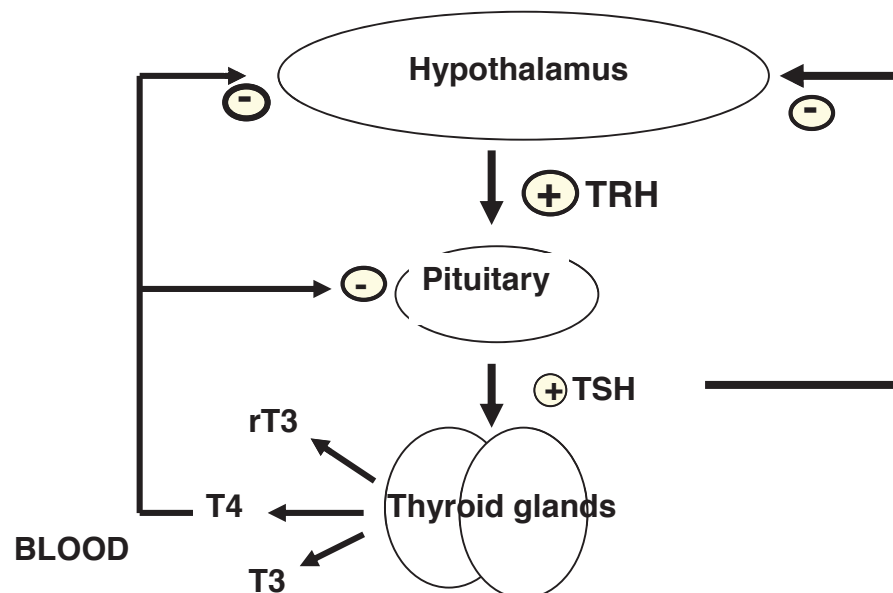
Figure 1.1.4: The hypothalamic-pituitary-thyroid axis. Thyroid secretion is controlled by thyroid-stimulating hormone (TSH) secreted by the anterior pituitary. TSH binds to the TSH receptor on the thyroid follicular cells and increases the production and secretion T_3 and T_4. The secretion of TSH from the pituitary is under control of the Thyroid releasing hormone (TRH) which is produced in the medial neurons of the paraventricular nucleus of the hypothalamus.

In the developing embryo, the thyroid follicular cells are recruited from endodermal origin from the foregut and migrate caudally from the pharynx, leaving behind a remnant called the thyroglossal duct. The C-cells of the thyroid will arise from the fourth branchial pouch. The thyroid migrates downward along the midline from the foramen caecum at the base of the primordial tongue to the base of the heart (Titlbach et al., 1987; Ohri et al., 1994; Patnaik et al., 2000; Reed et al., 2011)

Abnormal migration of the thyroid gland may result in ectopic thyroid tissue. Ectopic thyroid tissue has been identified in healthy cats in the tissues surrounding the tongue (Figure 1.1.5) (Patnaik et al., 2000; Reed et al., 2011) and in various other locations in hyperthyroid cats (Figure 1.1.6)(Naan et al., 2006; Harvey et al., 2009; Peterson & Broome, 2014).
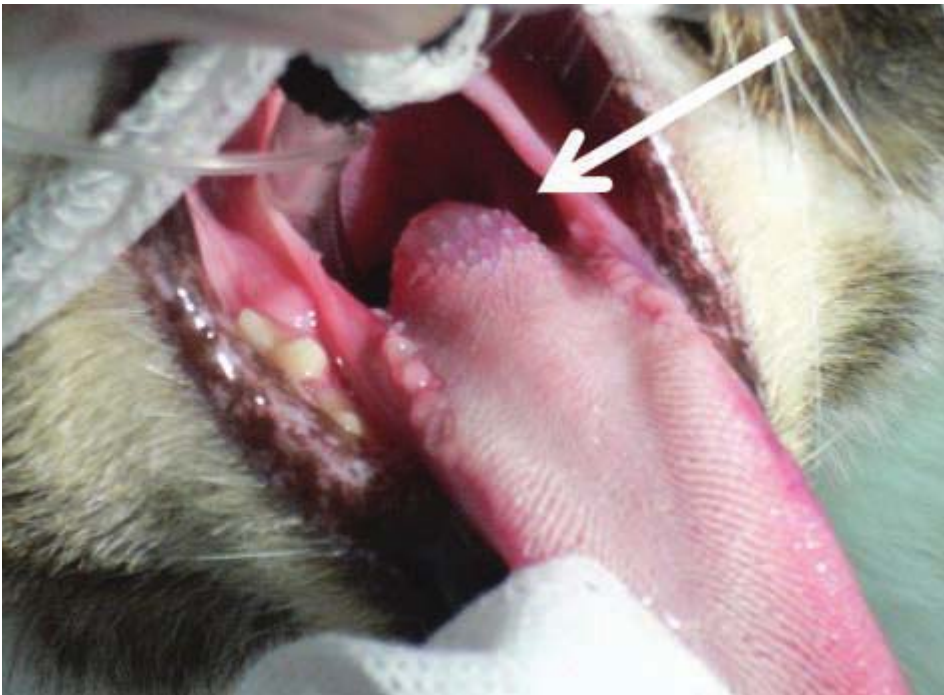
Figure 1.1.5: Photograph of a cystic lingual thyroid tissue (arrow) on the base of the tongue in a 6 year old, neutered, male domestic, shorthair cat. From Reed et al., 2011: *Journal of the American Veterinary Medical Association* 2011; 239:981–984.
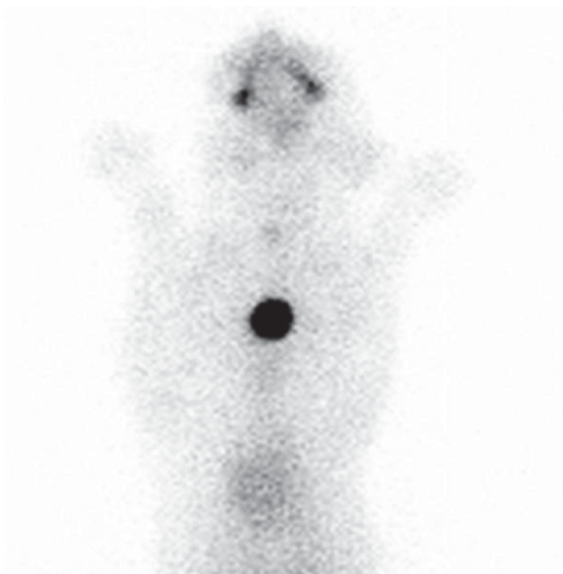
Figure 1.1.6: Thyroid scintigram of a hyperthyroid cat ectopic disease resulting from a single midline nodule in the intrathoracic (mediastinal) location. Notice that neither of the thyroid lobes in the normal cervical location is visible on this scintiscan.

From: (Peterson & Broome, 2014). *Veterinary Radiology and Ultrasound*, Vol. 56, No. 1, 2015, pp 84–95).

In many hyperthyroid cats, hyper functional thyroid tissue is also located in tissues outside of the thyroid gland (Naan et al., 2006; Harvey et al., 2009; Peterson & Broome, 2014). In a recent study ectopic thyroid tissue was found in 9/101 cats with hyperthyroidism (Naan et al., 2006). One study of scintigraphy scans of 120 hyperthyroid cats showed that that nearly 20% of cats have multiple areas of hyperplastic thyroid tissue or areas of thyroid tissue located in the thorax (Harvey et al., 2009). However, a recent, larger study of 2096 hyperthyroid cats undergoing scintigraphy identified thyroid tissue in the thoracic inlet in 282 (13.5%), and in the thoracic cavity in 115 (5.5%) and ectopic thyroid tissue (e.g. lingual or mediastinal) was diagnosed in 81 (3.9%) (Peterson & Broome, 2014).

1.3 Pathology

Benign, functional, adenomatous hyperplasia accounts for greater than 98% of cases of feline hyperthyroidism with the majority of the cases (>70%) having both thyroid lobes affected (Peterson et al., 1983). Thyroid carcinomas are the cause in 2-3% of cases of feline hyperthyroidism (Turrel et al., 1988; Naan et al., 2006). Adenomatous hyperplasia on histological examination reveals nodules of hyperplastic thyrocyte cells ranging in size from 1 mm- 3 cm (Peter et al., 1987). The disease in cats closely resembles human toxic nodular goitre or Plummer's disease in humans (Nayak & Hodak, 2007; Mooney, 2010; Peterson, 2014).

Toxic nodular goitre in people is the second leading cause of thyrotoxicosis in humans and is more common in geographic areas of iodine deficiency and people over the age of 50 years are most often affected (Nayak & Hodak, 2007). The most common cause of euthyroid goitre (thyroid enlargement) in people is iodine deficiency, with the prevalence of nodular thyroid disease being inversely proportional to iodine intake (Krohn et al., 2005). Thyroid nodular disease may be as high as 30-40% in women and 20-30% in men in iodine deficient areas (Krohn et al., 2005). Nodular thyroid disease is 5 to 15 fold more frequent in women, with the cause of the gender distribution being poorly understood (Krohn et al., 2005).

The exact aetiology of toxic nodular goitre in people and the progression of disease from euthyroid multinodular goitre to hyperthyroidism is still unknown. However, it has been postulated that the stimulus for thyroid hyperplasia can be iodine deficiency, nutritional goitrogens or autoimmunity. Thyroid hyperplasia will cause an increase in hydrogen peroxide (H_2O_2) production and free radical damage which in turn will increase DNA damage and cause a higher mutational load. Some of these spontaneous

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mutations will activate the cAMP cascade and activate TSH-R and $G_s\alpha$ mutations that further stimulate growth and function (Krohn et al., 2005; Paschke, 2011).

1.4 Aetiology of Hyperthyroidism in cats

A number of different causes have been postulated for the development of hyperthyroidism in cats which are broadly categorised into immunological, infectious, nutritional, environmental and genetic causes. No exact aetiology has been determined thus far (Martin et al., 2000; Edinboro et al., 2004; Olczak et al., 2005). Recent studies have focused on flame retardants such as polybrominated diphenyl ethers (PBDEs) which were introduced into homes some 30 years ago (Dye et al., 2007; Mensching et al., 2012; Dirtu et al., 2013; Chow et al., 2015). Interestingly, the time of introduction of fire retardants corresponds to the first cases of feline hyperthyroidism (Holzworth et al., 1980).

An in depth review of the aetiology of hyperthyroidism in cats is outside the scope of this review, and a number of recent reviews can provide further detail (Peterson & Ward, 2007; Peterson, 2014).

1.5 Clinical Presentation/features of Hyperthyroidism

Hyperthyroidism is a disease of middle aged to older cats, with a reported age range of 4-22 years (Peterson, 2000). The most common age of onset is 12-13 years of age and ninety-five percent of cats with the disease are over the age of ten (Peterson et al., 1983; Thoday & Mooney, 1992).

Two studies have reported a gender predilection for female cats (Edinboro et al., 2004; Olczak et al., 2005). Several reports indicate that purebred cats, especially Siamese and Himalayan cats have a lower chance of developing hyperthyroidism (Kass et al., 1999;

Olczak et al., 2005). Olczak et al (2005) found a predilection for hyperthyroidism in domestic short and long haired cats in New Zealand.

Over ninety percent of cats with hyperthyroidism present with a combination of clinical signs attributable to the increased secretion of thyroid hormone, such as polyuria, polydipsia, and polyphagia, weight loss in the face of a good appetite, hyperactivity, vomiting and diarrhoea. In the ten years since the disease was first diagnosed in cats, the severity of signs at first diagnosis has decreased (Broussard & Peterson, 1995) a feature which may be due to increased owner and veterinarian awareness of the disease and a diagnosis being made earlier before severe clinical signs are shown.

Weight loss occurs in nearly 80% of cats with hyperthyroidism (Peterson et al., 1983; Thoday & Mooney, 1992; Broussard & Peterson, 1995) and is usually gradual weight loss which can lead to severe cachexia. Polyphagia and vomiting is seen in around 50% of cats (Broussard & Peterson, 1995) and around 20% of cats may have periods of anorexia (Peterson et al., 1983). Polyuria and polydipsia occurs in around 40% of cats (Broussard & Peterson, 1995).

Physical examination findings include poor body condition and lack of muscle mass due to muscle catabolism, and often muscle weakness (Bucknell, 2000; Syme, 2007; Wakeling et al., 2008). Many cats are hyperactive which may be manifest as restlessness or even aggression (Labuc & Jones, 1988; Bucknell, 2000; Meeking, 2005). The changes in personality and behaviour are believed to be due to the increased metabolic rate driven by excessive thyroid hormone concentrations (Gunn-Moore, 2005; Syme, 2007). Cardiovascular signs have been associated with hyperthyroidism since it was first discovered (Fox et al., 1999). Tachycardia is the most common arrhythmia associated with the disease (Broussard & Peterson, 1995; Syme, 2007),

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hypertension (Meeking, 2005; Syme, 2007) and murmurs have also been commonly described (Thoday & Mooney, 1992).

Heart murmurs associated with hyperthyroidism vary from almost inaudible to significant. Heart murmurs are most commonly graded I to III/IV, and can be systolic or gallop murmurs, and are most often heart rate dependant (Thoday & Mooney, 1992; Syme, 2007). Echocardiography of cats with hyperthyroidism and heart murmurs has shown the likely cause of the murmur are rapid ventricular filling with a gallop rhythm (Syme, 2007) and dynamic left or right ventricular outflow tract obstruction for other murmurs (Rishniw & Thomas, 2002; Connolly et al., 2005).

With 10-20% of hyperthyroid cats having concurrent hypertension, it is important to examine the eyes for evidence of retinal changes which are indicative of hypertension – tortuous blood vessels, retinal tears or retinal detachment (Stepien, 2011).

1.5.1 Thyroid gland palpation

On physical examination, between 80-97% of cats with hyperthyroidism have an enlarged thyroid gland (Peterson et al., 1983; Thoday & Mooney, 1992; Broussard & Peterson, 1995). The thyroid gland can be enlarged in cats that do not have clinical or laboratory evidence of hyperthyroidism, enlargement which may be due to subclinical hyperthyroidism or other cervical masses (lymph nodes) or parathyroid hyperplasia seen with renal disease, or non- functional thyroid adenomas (Norsworthy et al., 2002; Wakeling et al., 2007). With enlargement, thyroid lobes can migrate towards the thoracic inlet or even into the mediastinum (Mooney, 2010).

To palpate the thyroid gland, two techniques have been described (Norsworthy et al., 2002; Paepe et al., 2008; Mooney, 2010). The first, or classical palpation technique; is where the cat is restrained in a sitting position with the front legs held still. The neck of

the cat is extended and the clinician's thumb and forefinger are placed on each side of the trachea sweeping down from the larynx to the sterna manubrium. Goitre is present when a mobile subcutaneous nodule is palpated as it slips through the clinician's fingers.

A second technique is semi-quantitative thyroid palpation (Norsworthy et al., 2002). For this technique, the clinician stands behind the cat, with the head of the cat elevated at 45°, while alternately moving the head left or right depending on which side is being assessed. The thyroid gland enlargement is scored between one and six – where a one is assigned to a thyroid lobe that is barely palpable, and a six to a lobe that is greater than 2.5 cm, and other values between proportionally assigned (Norsworthy et al., 2002)

Both of these palpation techniques were compared in a study, and both techniques had very good within- and good between -examiner agreement (the average weighted kappa-values within- and between-examiners were 0.864 and 0.644 for classical technique and 0.732 and 0.532 for the semi-quantative technique) (Paepe et al., 2008). The classical technique was preferred in this study, as there was more repeatable within- and between- examiner scores (Paepe et al., 2008).

1.6 Diagnosis of Hyperthyroidism

The diagnosis of hyperthyroidism, takes into account the history, physical examination, laboratory test results and thyroid function tests. Hyperthyroidism in cats is a disease of middle aged to older cats, therefore concurrent, non-thyroidal disease may also be present, which may alter the clinical presentation slightly. Hyperthyroid cats with concurrent renal, liver or neoplastic disease, may not have an increased appetite, however weight loss will still be a feature (Peterson, 2006a).

Between 15 to 50% of cats with hyperthyroidism will have concurrent renal disease, and clinical evidence for concurrent renal disease may not become apparent until after the cat is treated for hyperthyroidism, when the glomerular filtration rate and blood flow through the kidneys has returned to the euthyroid rate (Becker et al., 2000; Williams et al., 2010; Feeney et al., 2011). Thoracic radiographs, blood pressure measurement, electrocardiography and echocardiography may be required if heart disease is suspected from the physical examination.

A full over view of the diagnostic tests used for the diagnosis of hyperthyroidism is beyond the scope of this review, and a summary is provided. Extensive reviews of diagnostic testing for hyperthyroidism have recently been published (Peterson, 2013; Daniel & Neelis, 2014).

1.6.1 Complete blood count

The haematological changes in hyperthyroidism are not specific. An increase in packed cell volume, mean corpuscular volume and red blood cells counts and haemoglobin is reported in 47%, 44%, 21% and 17% respectively (Broussard & Peterson, 1995). The proposed mechanisms for these changes are increased oxygen consumption driving increased erythropoietin production and/or a thyroid hormone mediated beta adrenergic effect on bone marrow (Labuc & Jones, 1988; Shiel & Mooney, 2007). Anaemia in hyperthyroidism is rare, usually occurring in cases of severe hyperthyroidism as a result of bone marrow exhaustion, iron, or other micronutrient deficiency (Thoday & Mooney, 1992).

A stress leukogram is the most common finding with respect to white blood cells. There is generally a leukocytosis, mature neutrophilia, lymphopenia, eosinopenia and/or monocytopenia (Broussard & Peterson, 1995; Bucknell, 2000; Shiel & Mooney, 2007).

1.6.2 Serum chemistry

Elevations in at least one liver enzyme (alanine transaminase (ALT), alkaline phosphatase (ALP) and aspartate transaminase (AST)) is present in over 90% of hyperthyroid cats (Broussard & Peterson, 1995; Bucknell, 2000; Meeking, 2005). The increase in liver enzyme activity may occur due to hepatic dysfunction, hepatic hypoxia, or from the direct toxic effect of thyroid hormones on the liver (Labuc & Jones, 1988; Meeking, 2005). In euthyroid cats only the liver isoform of ALP is present in circulation however in hyperthyroid cats the bone isoform can also be isolated (Thoday & Mooney, 1992; Archer & Taylor, 1996; Meeking, 2005). The cause of the increased ALP has been proposed to occur due to increased bone marrow turnover (Peterson et al., 1983; Labuc & Jones, 1988) however the increased bone isoform of ALP suggests a change in the bone metabolism in hyperthyroid cats may only be one reason for the increase in ALP (Thoday & Mooney, 1992; Archer & Taylor, 1996).

Azotaemia is present in around 20% of cats with (Broussard & Peterson, 1995) with hyperthyroidism.

1.6.3 Urinalysis

Urinalysis changes in hyperthyroid are non-specific. Urine specific gravity generally is lower in hyperthyroid cats however the specific gravity is poorly correlated with the glomerular filtration rate (Langston & Reine, 2006; Syme, 2007). Proteinuria is a common finding. The mechanism for proteinuria is unclear but could result from either increased glomerular perfusion pressure or alterations in tubular protein handling (Syme, 2007). An elevated protein-creatinine ratio is also commonly seen (Langston & Reine, 2006) which may represent a balance between reduced serum creatinine concentration (low muscle mass) and increased renal protein excretion. Urinary tract

infections occur in around 12% of hyperthyroid cats and are not related to the low urine specific gravity (Mayer-Roenne et al., 2007).

1.6.4 Thyroid function tests

1.6.4a Thyroid hormone concentrations

Serum or plasma TT4 concentration is the best initial diagnostic test, as T4 is the main secretory product of the thyroid gland. Serum TT4 concentrations is an inexpensive test and is increased in around 91% of cases of cats with hyperthyroidism (Broussard & Peterson, 1995; Peterson et al., 2001). Fluctuations of the serum TT4 concentration can occur throughout the day therefore mildly hyperthyroid cats can have a serum TT4 concentration within the reference range (Peterson 1987). Non-thyroidal illness can also suppress serum TT4 concentrations into the reference range in mildly hyperthyroid cats. Severe non-thyroidal illness can suppress the serum TT4 concentrations into the mid to low end of the reference range in mildly hyperthyroid cats, however hyperthyroid cats should never have a serum TT4 concentration beneath the reference range. If hyperthyroidism is suspected and the serum TT4 concentration is within the reference range, repeating measurement of the serum TT4 concentration after treating non-thyroidal diseases or just repeating a serum TT4 concentration in 2 to 8weeks is recommended by Peterson (Peterson et al., 2001; Peterson, 2006a; Peterson, 2013).

1.6.4b Basal free thyroid hormone concentrations

Free T4 (fT4) measured by equilibrium dialysis is a more expensive assay method than measurement of serum TT4 concentration and is best used in conjunction with serum TT4 concentration when there are clinical signs of hyperthyroidism with the TT4 concentration is in the reference range. Free T4 is increased in 98.5% of hyperthyroid cats, and all cats with increased TT4 have an increased fT4 (therefore little information is added if the TT4 is increased) (Peterson et al., 2001). Six to twelve percent of

euthyroid sick cats can have increased fT4. A low TT4 and high fT4 concentration is usually associated with non-thyroidal illness (Mooney et al., 1996; Peterson et al., 2001).

1.6.4c Thyroid hormone suppression test

Feline thyroid stimulating hormone (TSH) assays have not been commercially developed, however the use of canine TSH assays may have a role in aiding the diagnosis of hyperthyroidism in cats (Wakeling et al., 2007; Wakeling et al., 2008; Wakeling et al., 2011).

1.6.4d Dynamic thyroid testing

In the majority of cats with a thyroid nodule and clinical signs of hyperthyroidism, if the serum TT4 concentrations are normal, then repeat TT4 concentrations in a few weeks or measurement of Free T4 concentrations will confirm the diagnosis of hyperthyroidism (Peterson, 2013). Dynamic thyroid testing such as Thyrotropin-releasing (TRH) hormone stimulation test or T3 suppression tests (Peterson et al., 1990; Peterson et al., 1994) have previously been recommended for confirming a diagnosis of hyperthyroidism in cats with equivocal serum thyroid hormone results. Dynamic thyroid testing is rarely considered necessary, as free T4 or repeating measurement of TT4 concentrations or thyroid scintigraphy will often result in a diagnosis of hyperthyroidism (Peterson, 2013).

1.6.5 Imaging of the thyroid gland

1.6.5a Thyroid radionuclide uptake

Scintigraphy is a method that can be used for determining location of thyroid tissue and the size of thyroid masses (Daniel et al., 2002; Broome, 2006). Several choices of radioisotope are available for thyroid imaging (^{131}I and ^{123}I and Technetium pertechnetate (TcO$_4^-$), ^{131}I is more commonly used therapeutically (Mooney, 1994; Slater et al., 1994; Guptill et al., 1995; Peterson, 2006b). Technetium pertechnetate (TcO$_4^-$) is absorbed by thyroid follicular tissue and is most commonly used for thyroid scintigraphy (Daniel et al., 2002; Feeney & Anderson, 2007). Technetium pertechnetate is also absorbed by gastric mucosa and salivary gland tissue and a comparison between salivary gland and thyroid density is used to quantify thyroid size and density. Normal cats have a thyroid-to-salivary ratio of 0.87:1 (range 0.6:1- 1.03:1) (Daniel et al., 2002; Henrikson et al., 2005; Daniel & Neelis, 2014).

Scintigraphy is particularly useful in locating ectopic thyroid tissue. A recent study of over 2096 scinitigraphy scans of hyperthyroid cats, identified ectopic thyroid tissue in approximately 4% of cases, whereas previous, small studies have identified ectopic tissue in nearly 25% of hyperthyroid cats (Harvey et al., 2009; Peterson & Broome, 2014). Around 70% of cats with have bilateral thyroid gland adenomatous hyperplasia, making scintigraphy crucial for surgical planning (Naan et al., 2006; Peterson & Broome, 2014). The size and density of the thyroid tissue can also allow for individual dose calculation for I^{131} treatment in affected cats (Wisner et al., 1994). A good review of thyroid scintigraphy has recently been published, and further discussion on scintigraphy is outside the scope of this review (Daniel & Neelis, 2014).

1.6.5b Thyroid ultrasound

There is limited information available on ultrasonography of the thyroids in normal and hyperthyroid cats. In humans, ultrasound of the thyroid gland is used routinely to evaluate patients with hyperthyroidism and thyroid nodules (Barraclough & Barraclough, 2000). Ultrasonography has also been used in dogs to discriminate healthy, euthyroid sick and hypothyroid dogs (Bromel et al., 2005; Reese et al., 2005).

In cats, one study evaluated six healthy cats and 14 hyperthyroid cats comparing thyroid ultrasonography with thyroid $^{99M}TcO_4^-$ scintigraphy (Wisner et al., 1994). An 85.7% agreement was found between scintigraphy and ultrasonography in differentiating cats with hyperthyroidism from the healthy cohort. A recent ultrasonographic study evaluated hyperthyroid cats, pre and post treatment with radioactive iodine (Barberet et al., 2010). Hyperthyroid cats had unilateral or bilateral thyroid lobes with increased volume and vascularity, as seen by the Power Doppler. Six months after treatment with radioiodine, the thyroid vascularity decreased as did the volume of the thyroid gland (Barberet et al., 2010). Ultrasound identification of enlarged thyroid gland(s) also correlates well with the palpation score in normal and hyperthyroid cats (Paepe et al., 2008).

1.7 Therapy of Hyperthyroidism in cats

With over 98% of feline hyperthyroidism due to benign disease, the prognosis for this disease is excellent with a number of effective therapy options. There are four treatment options for hyperthyroidism: thyroidectomy, radioactive iodine, nutritional therapy with low iodine diets and medical therapy with anti-thyroid drugs such as carbimazole (Neomercazole® or Vidalta®) or methimazole (Tapazole®) (Peterson et al., 1988; Peterson & Becker, 1995; Bruyette, 2004; Gunn-Moore, 2005; Peterson, 2006b; van Hoek et al., 2007). All treatment options need to be discussed and the pros and cons for each evaluated for the cat and its owner. Which treatment is selected depends on a number of factors including: concurrent disease (chronic kidney disease, diabetes mellitus), age of the cat, cost, surgical skill, availability of nuclear medicine facilities and the owner's informed opinion.

Radioactive iodine therapy is considered the gold standard for treatment of hyperthyroidism; however, many pet owners prefer medical or nutritional management (Peterson & Becker, 1995; Peterson, 2006b; Caney, 2013; van der Kooij et al., 2014). A recent survey of 630 general practitioners in the United Kingdom, revealed that oral medication was the most commonly preferred treatment option (65.7% of respondents), followed by thyroidectomy (27.5%) and then radioiodine (5.5%) (Higgs et al., 2014). However, if cost of treatment was not a concern more respondents selected radioiodine (40.5%, $p<0.001$) (Higgs et al., 2014). A brief overview of all therapeutic options is included in this review.

1.7.1 Radioactive iodine (^{131}I)

Radioactive iodine (^{131}I) has the fewest side effects and is the most efficacious of the treatments for feline hyperthyroidism, however availability can be limited, upfront costs can be high, clients may not like radiation therapy, and cats with concurrent renal

disease might not be suitable candidates (Peterson & Becker, 1995; Langston & Reine, 2006; Peterson, 2006b; Syme, 2007).

The only known function for stable iodine is for thyroid hormone synthesis, therefore radioactive iodine is actively accumulated in the thyroid gland. The ^{131}I concentrates preferentially in the hyperactive or neoplastic tissue and destroys it (Peterson & Becker, 1984; Peterson & Becker, 1995). Radioactive iodine, (^{131}I) has a radiation half-life of 8 days and emits both β-particles and γ-radiation. The β-particles cause 80% of the tissue damage, however only travel 2mm into the tissue, therefore are locally destruction to hyperplastic tissue. Conversely, the β-particles will be sparing of local hypoplastic tissue and other structures such as the parathyroid glands (Peterson, 2006b).

Radioactive iodine (^{131}I) can be administered orally, subcutaneously or intravenously. The subcutaneous dose is safer, simple and less stressful to the cat. The dose of ^{131}I to treat cats with hyperthyroidism is controversial (Peterson, 2006b). Fixed doses (148–185 mBq, 4–5 mCi) (Craig et al., 1993; Milner et al., 2006), doses determined by tracer kinetic studies (Broome et al., 1988; Malik et al., 1994) and doses using a combination of clinical signs and fixed dose have been used (Mooney, 1994; Peterson & Becker, 1995).

Survival time of cats treated with ^{131}I range from two weeks to seven years with a median survival time of 24 months (Peterson & Becker, 1995).

The major disadvantages of ^{131}I is that there are special licensing requirements required to administer and house the cats after treatment to ensure radiation exposure to humans is minimised. These requirements limit the availability of this treatment and increase the costs of this treatment.

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1.7.2 Surgery

Surgical removal of the thyroid glands is an effective and relatively quick treatment for hyperthyroidism. However it must be remembered that 70% of cats with hyperthyroidism have bilateral disease (Peterson & Broome, 2014), so surgical cure may not be permanent if a unilateral thyroidectomy is performed. In the hands of a skilled surgeon, unilateral thyroidectomy is safe and effective, although recurrence of clinical signs occurs in around 5% of cases.

Serum calcium concentrations are monitored for a few days post-surgery as hypocalcaemia resulting from hypoparathyroidism, is the most significant postoperative complication. Hypocalcaemia is rare and usually only occurs after bilateral thyroidectomy when both parathyroid glands have been removed or if the parathyroid glands are damaged. The cat is treated with calcium and Vitamin D if hypocalcaemia develops (Birchard, 2006; Naan et al., 2006).

Recurrence of hyperthyroidism is mostly due to the secretion of thyroid hormones from ectopic thyroid tissue. Thyroid scintigraphy is recommended prior to surgery to identify ectopic tissue, present in 4–20% of hyperthyroid cats (Harvey et al., 2009; Peterson & Broome, 2014). Medical management is also recommended prior to surgery to reduce/stabilise the clinical signs of hyperthyroidism and the potential thyrotoxic cardiac effects (Naan et al., 2006).

Serum thyroid concentrations should be measured after surgery, to ensure the cat does not develop hypothyroidism, as asymptomatic hypothyroidism can progress chronic kidney disease in cats (Williams et al., 2010).

1.7.3 Nutritional therapy

In 2011 in the United States of America, and in 2013 in New Zealand, Hill's Prescription Diet y/d Feline Thyroid Health was released as an alternative therapy to manage hyperthyroidism. The diet is iodine restricted, and the therapeutic effect is based on the fact that iodine plays an essential role in the synthesis of T3 and T4 (Figure 1.1.3). Abstracts presented at the American College of Veterinary Internal Medicine (ACVIM) Forum in 2011, concluded that dietary restriction of iodine was an effective method for decreasing serum TT4 concentrations in cats with hyperthyroidism and that the maximum iodine concentration required in foods to control hyperthyroidism was 0.39 ppm (Melendez L.M. et al., 2011a; Melendez et al., 2011b).

In 2014, a study of client owned hyperthyroid cats fed Hill's Prescription Diet y/d Feline Thyroid Health for eight weeks was published (van der Kooij et al., 2014). After eight weeks 51/68 cats had a TT4 concentrations in the normal reference range, nine of the 17 cats with increased serum TT4 concentrations had poor owner compliance or palatability issues, compared to nine of the 51 with normal serum TT4 concentrations (van der Kooij et al., 2014). Conclusions from this study were that Hill's Prescription Diet y/d Feline, was a valuable management option to reduce TT4 concentrations within 4 weeks in client owned hyperthyroid cats, providing that the cat exclusively ate the iodine restricted diet and that the owner is compliant with feeding the diet. Further studies are required to determine the long term efficacy of the Hills Y/d diet in hyperthyroid cats and also the effect of feeding the diet before radioactive iodine therapy.

1.7.4 Medical therapy

<u>*Introduction*</u>

The use of carbimazole or methimazole (United States Adopted Name [USAN])/

thiamazole (International Nonproprietary Name [INN]) to treat cats with

hyperthyroidism has been standard practice since the discovery of the disease in the

early 1980s (Peterson, 1984a; Peterson et al., 1988; Trepanier, 1990; Trepanier et al.,

1991a; Mooney et al., 1992; Peterson & Aucoin, 1993; Bucknell, 2000). Carbimazole is

a pro-drug of methimazole that is used as tablets to treat hyperthyroid cats in Europe,

Australia and New Zealand, (Mooney et al., 1992; Bucknell, 2000; Frenais et al., 2008)

In New Zealand, the use of carbimazole to treat feline hyperthyroidism is off label, as

the drug is not registered for veterinary use. In Europe, a once daily long acting

carbimazole tablet (Vidalta®) has been registered for veterinary use, and the Food and

Drug Administration (FDA) recently approved methimazole for the treatment of feline

hyperthyroidism in the USA (Frenais et al., 2008; FDA, 2009; Frenais et al., 2009).

Long term medical therapy is indicated in cats where surgery or radioactive iodine

treatment is not possible. Medical management is also recommended prior to surgery to

promote a euthyroid state and reverse the hyperthyroid condition, creating a more stable

patient for anaesthesia and surgery. Medical management is also recommended prior to

radioactive iodine administration to assess if renal function is impaired (Trepanier,

2007). A study has also shown that there is a greater reduction in serum TT4

concentrations in cats that are treated with radioactive iodine that have been pre-treated

with methimazole than those cats that have not been treated with methimazole (Feeney

et al., 2011).

Other medical treatments for feline hyperthyroidism, such as Propylthiouracil (PTU)

and iodinated radiographic contrast agents (Ipodate and Ipanoic acid) have been trialled

in cats with minimal success. PTU was widely used to treat hyperthyroidism in people and has the additional advantage of inhibiting peripheral T3 conversion, however the drug has serious side effects in cats such as hepatopathy, Coombs positive haemolytic anaemia and thrombocytopenia, so is no longer recommended (Peterson, 1981). Sodium ipodate is effective in the treatment of some hyperthyroid cats, but is not available as a commercial preparation (Murray & Peterson, 1997). A similar compound, ipanoic acid, has been evaluated in 11 hyperthyroid cats and showed efficacy in rapidly decreasing serum thyroxine concentrations, however this agent is not recommended for long term management (Gallagher & Panciera, 2011). The remainder of this review will focus on carbimazole and methimazole for the medical therapy of hyperthyroidism.

Background on carbimazole and methimazole

Carbimazole and methimazole (USAN) / thiamazole (INN) are pharmacological agents for the treatment of hyperthyroidism in cats. Throughout this review, the term methimazole is used, as this term is the most common term reported in the veterinary literature.

Methimazole (Tapazole®, Felimazole®) is the drug used to treat hyperthyroidism in cats in North America, with carbimazole (Neo-Mercazole®, Vidalta®), a pro-drug of methimazole, used in Europe and the Southern hemisphere (Peterson et al., 1988; Mooney, 2002; FDA, 2009; Frenais et al., 2009).

Carbimazole was initially developed with a view that it would have a longer antithyroid action than methimazole (Lawson et al., 1951), however it was soon found that the *in vivo* action of carbimazole was due to its rapid metabolism to methimazole (Nakashima & Taurog, 1979). In cat plasma carbimazole is rapidly converted to methimazole by an enzymatic process, and all the resultant antithyroid effects are due to methimazole

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(Peterson & Aucoin, 1993). Carbimazole has a molecular weight of 186 and methimazole a molecular weight of 114 (Figure 1.1.7) and these drugs are considered equipotent on a molar rather than a weight basis. To calculate the equimolar equivalent dose of methimazole, the carbimazole dose should be multiplied by 0.61 (Jansson et al., 1983).

methimazole   carbimazole
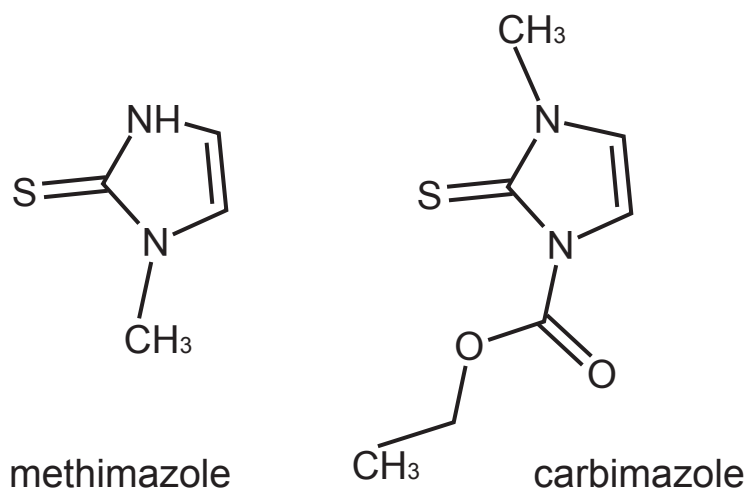
Figure 1.1.7: The structure of methimazole and carbimazole. The molecular weights are 114 and 186 respectively (from Jansson et al 1983).

Mode of action of methimazole

Methimazole is actively concentrated in the thyroid gland and the primary action is to inhibit thyroid hormone synthesis, by interfering with thyroid peroxidase-mediated iodination of tyrosine residues, mono-iodotyrosine (MIT) and, diiodotyrosine (DIT) in thyroglobulin and preventing the formation of thyroxine (T4) and tri-iodothyronine (T3) (Cooper, 2005) (step 3 and 4 in Figure 1.1.3). In the presence of methimazole, the drug is an alternative substrate for the iodinating intermediate and diverts oxidized iodide away from hormone synthesis. Formation of inactive methimazole products such as methylimadazole occurs.

Methimazole also has immunomodulation effects by reducing Major histocompatibility (MHC) class-I expression and inhibiting interferon-gamma induced expression of the

MHC class-II genes in thyroid epithelial cells (Giuliani et al., 2010). Human patients that receive methimazole as a treatment for the immune mediated hyperthyroid condition Grave's disease, show a decrease in serum concentrations of antithyrotropin-receptor antibodies and other important immune molecules such as intracellular adhesion molecule 1, soluble interleukin-2 and interleukin-6 receptors have also been shown to decrease over time (Cooper, 2005).

The next section will discuss in further detail the pharmacokinetics and efficacy of carbimazole and methimazole.

2.0 PHARMACOKINETICS OF METHIMAZOLE AND CARBIMAZOLE IN CATS

There are no significant differences between the pharmacokinetics of methimazole and carbimazole in cats (Trepanier et al., 1991a) (Peterson & Aucoin, 1993). This is an expected finding as previously stated, the *in vivo* action of carbimazole is due to its rapid metabolism to methimazole (Nakashima & Taurog, 1979). After the oral administration of 5 mg of carbimazole to nine cats, serum concentrations of methimazole increased and serum concentrations of carbimazole were undetectable in eight cats (Peterson & Aucoin, 1993). The serum half- life (measured as methimazole) after oral administration of 5 mg of carbimazole was 4.4 hours and after administration of 5 mg of methimazole between four to six hours (Trepanier et al., 1991a) (Peterson & Aucoin, 1993). The mean absolute bioavailability of methimazole in healthy (76 to 81%) and hyperthyroid (81%) cats is similar to the reported absolute bioavailability of controlled release carbimazole in healthy cats (88%) (Trepanier et al., 1991a; Trepanier et al., 1991b; Frenais et al., 2008). In hyperthyroid cats, there is a trend for faster elimination of methimazole, however this more rapid elimination is not considered important in regard to therapy (Trepanier et al., 1991b).

2.1 Efficacy of oral methimazole & carbimazole – cat studies

2.1.1 Carbimazole oral tablets

There are only two clinical studies on the use of carbimazole to treat feline hyperthyroidism in a total of 75 cats, with only 22 cats treated with carbimazole for longer than four weeks (Mooney et al., 1992; Bucknell, 2000). An initial dose of 5 mg three times a day was recommended for two to three weeks. Euthyroidism with serum thyroid hormone concentrations occurred rapidly within a mean of six days. Long term therapy was recommended at 5 mg twice daily, with more than 90% of cats remaining

euthyroid (Mooney et al., 1992). In this study, 18% of cats had adverse effects associated with carbimazole including anorexia, vomiting, depression, lymphocytosis and leukopenia.

Recently, a once daily formulation of carbimazole (Vidalta®) to treat hyperthyroidism in cats has been licensed for use in cats in Europe and New Zealand. The clinical efficacy of this drug was determined over 53 weeks in 44 client owned hyperthyroid cats (Frenais et al., 2009). Twenty cats completed the study. The median dose of carbimazole at the end of the study was 15 mg once a day. The median serum TT4 concentration had decreased to 33 nmol/L after 10 days and 21 nmol/L after 53 weeks. However, between 24-30% (8/34, 12/40) of cats did not have a serum TT4 concentration less than 50 nmol/L at each of the recheck dates. At three weeks, 64% (24/36) cats were euthyroid, compared to 94% of cats at 53 weeks (17/18) (Frenais et al., 2009). Eighty-five percent of owners reported the ease of tablet administration throughout the study. A direct comparison between the sustained release formulation and the conventional formulation has not been performed.

Adverse reactions were seen forty-six times, with the most frequently reported signs being vomiting, diarrhoea, reduced appetite/anorexia, bodyweight loss and skin abnormalities (Frenais *et al*. 2009). Carbimazole was thought to be the possible cause of adverse reactions in 20 cases (44%), which were mostly gastrointestinal signs. Carbimazole therapy was stopped prematurely in 8 (18%) cats with adverse reactions (Frenais *et al*. 2009).

2.1.2 Methimazole oral tablets

Initially, a dose of 10 to15 mg divided once to twice daily was recommended for the treatment of hyperthyroid cats (Peterson et al., 1988). As cats are now diagnosed with

less severe clinical signs, and hence treated earlier in the disease process, a dose of 2.5 to 5 mg once or twice daily will be efficacious to treat most hyperthyroid cats (Trepanier et al., 2003; Rutland et al., 2009). A study showed there was no significant relationship between serum thyroid hormone concentrations and the time after administration of methimazole or the *dosing* interval, therefore the timing of blood sampling for thyroxine concentrations after oral methimazole administration is not a significant factor (Rutland et al., 2009). Clinical side effects after methimazole administration can occur in 18% of cats including anorexia (11%), vomiting (11%), lethargy (9%), facial excoriations (2%), bleeding (2%) and icterus (1.5%) (Peterson et al., 1988). Positive antinuclear antibodies (ANAs) have been documented in more than 20% of treated cats but their clinical significance is uncertain (Peterson et al., 1988).

As carbimazole is rapidly converted to methimazole, cats that have adverse reactions to methimazole requiring withdrawal of therapy should not be treated then with carbimazole (Trepanier, 2007). Table 1.2.1 (modified from (Scott-Moncrieff, 2014)) summarizes the literature on the side effects of oral methimazole and carbimazole in cats.

In a recent survey (Higgs et al., 2014) conducted over 12 months, of 603 United Kingdom general veterinarians, sustained-release carbimazole tablets was favoured by 311 of the respondents (51.6%), methimazole by 244 (40.5%) and 39 (6.5%) had no specific preference. The unlicensed, human form of carbimazole tablets (Neomercazole; Roche Products) was chosen by 9 (1.5%) respondents, which would be in accordance with British law to use a registered veterinary product. The following side effects were observed by the percentage of vets shown (Higgs et al, 2014): Vomiting 69%, anorexia 47%, facial pruritus 44.8%, azotaemia 22.7%, anaemia 11.8%, leukopenia 10.9%,

hepatic damage 9.6%, neutropenia 8.4%, thrombocytopenia 8.4%, lymphadenopathy

4.7% and sudden death 0.9%.

Several case reports have documented other side effects of methimazole. Myasthenia

gravis has been reported in a cat treated with transdermal methimazole (Bell et al.,

2012). Lymphadenomegaly has been documented with both carbimazole and

methimazole administration in cats (Niessen et al., 2007; Snead et al., 2013) and

pyogranulomatous mural folliculitis was also seen in one cat (Castro Lopez et al.,

2014).

Table 1.2.1: Adverse reactions associated with drugs used therapeutically in feline

hyperthyroidism. Adapted from Scott-Moncrieff, J.C. 2014

Drug (reference)	Reaction	Approximate percentage of cats affected	Time at occurrence	Treatment required
Methimazole (Peterson et al 1988)	Vomiting, anorexia, depression	11	< 4 weeks	Usually transient, decrease dose
	Eosinophilia, leukopenia, lymphocytosis	15	< 8 weeks	Usually transient
	Self-induced excoriations	2	< 4 weeks	Withdrawal and glucocorticoid therapy
	Agranulocytosis, thrombocytopenia	< 5	< 3 months	Withdrawal and symptomatic therapy
	Hepatopathy (anorexia,↑alanine aminotransferase, alkaline phosphatase)	< 2	< 2 months	Withdrawal and symptomatic therapy
	Positive antinuclear antibody	> 20	> 6 months	Decrease daily dosage
	Acquired myasthenia gravis	Rare	< 16 weeks	Withdrawal and appropriate treatment

Drug (reference)	Reaction	Approximate percentage of cats affected	Time at occurrence	Treatment required
Carbimazole conventional (Mooney 1992, Bucknell 2000)	Vomiting, anorexia, depression	10	< 3 weeks	Usually transient, decrease dose
	Eosinophilia, leukopenia, lymphocytosis	5	< 2 weeks	Usually transient
	Self-induced excoriations	Rare	< 4 weeks	Withdrawal and glucocorticoid therapy
Carbimazole sustained release (Frenais 2009)	Vomiting	32		Usually transient, may require withdrawal
	Diarhoea	11		Usually transient, may require withdrawal and symptomatic therapy
	Anorexia	9		May require withdrawal and symptomatic therapy
	Eosinophilia, leukopenia, lymphocytosis	7		Usually transient
	Pruritus	7		Withdrawal and glucocorticoid therapy

2.2 Monitoring of medical therapy

The recent survey of 603 veterinarians by Higgs et al (2014), asked questions on the frequency of monitoring parameters for recently diagnosed hyperthyroid cats treated medically and 228 (37.8%) respondents stated that they followed a practice protocol (of which 204 were happy with the protocol), 224 (37.1%) designed their own monitoring protocols and 137 (22.7%) based their monitoring protocols on published medication datasheet advice. The most common parameters to monitor were body weight, serum TT4 concentrations and renal biochemistry. Body weight was the most commonly chosen parameter with 321 (53.2%) respondents weighing the cat every three months, 220 (36.5%) every six months and seven (1.2%) annually. Assessment of serum TT4 concentrations would be measured every three months by 155 (25.7%) respondents, every six months by 310 (51.4%) respondents and annually by 45 (7.5%). Blood pressure was measured in the first month by 251 (41.6%) and recommended to be measured every three months by 91 (15.1%), every six months by 144 (23.9) or annually by 72 (11.9%).

An excellent guideline on the best practice for the pharmacological management of hyperthyroidism in cats has recently been published (Daminet et al., 2014), and should be referred to for further information. These guidelines include monitoring recommendations, which according to the survey by Higgs et al, many veterinarians are not actually performing.

Baseline monitoring parameters that are recommended include (modified from Daminet et al 2014):

- A thorough case history and physical examination (including cervical palpation and emphasis on cardiac assessment);

- Bodyweight and body condition score ;

- Blood pressure measurement to establish baseline and to familiarise the cat to the procedure;

- Ophthalmological examination (fundic examination by indirect ophthalmoscopy to assess evidence of hypertensive retinopathy);

- Circulating serum TT4 concentration;

- Complete blood cell count (CBC);

- Blood biochemistry, including a liver profile; and

- Urinalysis: urine-specific gravity (USG), dipstick analysis and sediment examination as a minimum, urine culture is ideal.

The history, body weight, serum TT4 concentrations, renal profile and blood pressure should then be assessed two to three weeks after therapy is started until the cat is euthyroid, then at three months and then every six months. The authors of these guidelines recommend a serum TT4 concentration within the lower half of the reference interval set by the reference laboratory (Daminet et al., 2014).

2.3 Transdermal application of methimazole/carbimazole in cats

A recent survey in the United Kingdom of 111 cat owners, reported 62% of owners said that administering pills *per os* to their cat twice a day was not a problem (Caney, 2013). However, many cat owners find it difficult to medicate their cats and many compounding pharmacies have started formulating drugs into gels to be applied to the inner side of the ear of the cat and the drug is thought to be absorbed through the skin to allow systemic action (Marks & Taboada, 2003). Methimazole is one of the drugs that has been applied to the inner pinna of cats in Pluronic® lecithin organogel (PLO) gel.

Pharmacokinetic studies of methimazole in PLO gel showed that methimazole in this formulation was poorly absorbed when applied to the inner pinna, with only two of five cats having measurable serum methimazole concentrations in the first 24 hours (Hoffman et al., 2002; Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006). The first reports of methimazole in PLO gel in hyperthyroid cats were two clinical case series (Hoffmann et al., 2003; Lecuyer et al., 2006). Transdermal methimazole in PLO gel applied twice daily was shown to have short term (4-8 weeks) efficacy for the treatment of hyperthyroid cats after repeated applications (Hoffmann et al., 2003; Lecuyer et al., 2006).

The first clinical case series reported was by Hoffman et al (2003), a retrospective case series of 13 hyperthyroid cats treated with methimazole in PLO gel at a dose ranging from 2.5 mg per cat every 24 hours to 10 mg per cat every 12 hours. Due to the retrospective nature of this study, there was no consistent re-evaluation protocol. The mean time for recheck 1 was 4.3 weeks, and mean for recheck 2 was 5.4 months. Significant decreases in serum TT4 concentrations were measured at recheck 1 (mean 39.57 nmol/L) and at recheck 2 (mean 36.71 nmol/L) compared to the pre-treatment concentrations (mean 97.5 nmol/L). Adverse effects were not reported. This study

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supported the application of methimazole in PLO gel, and indicated that further prospective studies and pharmacokinetic trials were required.

The second study, was a prospective clinical case series by Lecuyer et al (2006), of 13 newly diagnosed cats with hyperthyroidism, treated with methimazole in PLO gel at a dose of 5 mg applied to the inner pinna every 12 hours for 28 days. The cats were evaluated at 14 and 28 days after treatment commenced. Only ten cats completed the trial. A significant decrease in serum TT4 concentrations, was seen in all cats. The mean serum TT4 concentrations at day 14 (27.44 nmol/L) and day 28 (14.63 nmol/L) were lower, ($p<0.0001$), compared with the serum TT4 concentrations at the start of the trial (97.31 nmol/L). However, as a vehicle for methimazole, PLO gel had problems with drug precipitation. One third of owners reported development of a non-homogenous texture to the gel which could have resulted in variation in the concentrations of methimazole delivered at each dose (Lecuyer et al., 2006).

A randomized trial that compared oral methimazole to methimazole in PLO gel applied to the inner pinna, found methimazole in PLO gel to be less efficacious than oral treatment with methimazole, (only 14/21, 67% euthyroid by 4 weeks) compared with oral methimazole (9/11, 82% euthyroid by 4 weeks) although this difference was not significant ($p=0.36$) (Sartor et al., 2004). Fewer gastrointestinal side effects (1/27, 4% of cats) were seen in the methimazole in PLO group compared to 24% (4/17) of cats in the oral methimazole group (Sartor et al., 2004). The dose of methimazole in PLO gel was 2.5 mg administered every 12 hours. No differences in the incidence of other common side effects such as facial excoriation, neutropenia, thrombocytopenia, or hepato-toxicity were detected between the two groups. The lower bioavailability of methimazole in PLO gel was provided as the reason for the lower efficacy compared to the oral route.

A long term clinical case series (median follow up of 22 months) of methimazole in PLO gel has recently been published, reporting that transdermal methimazole is a safe option for the long term therapy of hyperthyroid cats, however the dose of methimazole may need to be increased over time (Boretti et al., 2014). The median dose of methimazole in PLO gel at 24–36 months was 7 mg (range 2.5–10) was higher than the starting dose of 5 mg (range 2.5–5) (p<0.05). The higher dose may be required due to the increased methimazole requirements of cats resulting from continued growth of the thyroid adenoma which in some cats may eventually become a thyroid carcinoma, an event that has been suggested by Dr Mark Peterson (Peterson, 2012). Owner compliance was variable in this study, with many owners admitting to not treating the cat regularly and some to stopping therapy altogether, therefore one conclusion from the study was that despite the convenience of transdermal application, owner compliance still needs to be monitored regularly to ensure continuation of daily treatment (Boretti et al., 2014).

Boretti (2013) also published a study on the length of time that serum TT4 concentrations are suppressed after once or twice daily application of methimazole in PLO gel. Prolonged suppression of serum TT4 concentrations were observed, therefore the conclusion from the study was that the timing of blood sampling for serum TT4 concentrations was not critical when monitoring patients treated with methimazole in PLO gel (Boretti et al., 2013) (Note : this study was published after the clinical trial performed in **Chapter 4**).

There is only one study on the use of carbimazole in PLO gel, published in Dutch in 2006 (Note : The Dutch study was simultaneous to the study being undertaken for this thesis; **Chapter 2**) (Buijtels et al., 2006). Buijets *et al* (2006) enrolled 13 cats with hyperthyroidism, nine of which completed the trial to determine the efficacy of

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carbimazole in PLO gel applied to the inner pinna at 4, 8 and 12 weeks. A dose of 4 to 17 mg twice a day was required to restore the serum TT4 concentration to within the reference range. The conclusions from this study was that the twice daily administration of carbimazole to the inner pinna of cats was an effective therapy for hyperthyroidism in cats (Buijtels et al., 2006).

The advantages of transdermal administration of methimazole to cats includes fewer gastrointestinal side effects, perceived ease of administration by owners and perhaps increased adherence to the medication, however compliance may waver over time (Marks & Taboada, 2003; Sartor et al., 2004). Disadvantages of transdermal methimazole include erythema of the ear, increased cost of the medication and an unknown safety and unproven stability of the drug and gel agent (Trepanier, 2007).

Further research in the field of percutaneous absorption via the pinna is required. Most of the literature published are clinical case series (Hoffmann et al., 2003; Lecuyer et al., 2006; Boretti et al., 2014). Only one randomized trial (Sartor et al 2004), compared the methimazole *per os* with methimazole applied to the inner pinna, no trials have compared transdermal methimazole with other treatment options such as, nutritional therapy, surgery or radioactive iodine. When deciding on a treatment option for hyperthyroidism, a clinician must consider all the relevant client and patient factors, and also the possible necessity for combination of therapies (i.e. pre-treatment with methimazole prior to surgery or radio-active iodine).

Carbimazole (log P 1.35)[a] is a more lipid soluble pro-drug of methimazole (log P 0.75), therefore carbimazole should theoretically be more completely absorbed by the transdermal route than methimazole. Further research is required to determine if PLO gel is the most appropriate vehicle for the percutaneous delivery of carbimazole and

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[a] The log P value, or partition co-efficient, refers to the lipophilicity of the unionised species, and lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution in a biphasic system (e.g. partition coefficient in octanol-water).

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methimazole as PLO gel might not be the most suitable vehicle for these lipid soluble drugs (Mills & Cross, 2006b). A suitable vehicle should be sufficiently soluble to contain the active drug without the drug precipitating out of solution (Mills & Cross, 2006b). Currently it is unknown how much carbimazole or methimazole penetrates the ear of cats and whether PLO is the most appropriate vehicle, and whether methimazole residues in the ear could cause potential harm to other in contact cats or humans in the household.

2.4 Safety of Methimazole applied topically to the skin

Methimazole has recently been used topically to treat melasma in people (Kasraee et al., 2005; Kasraee et al., 2008; Malek et al., 2013). Topical methimazole was first shown to cause depigmentation of the ears of brown guinea pigs in a pilot trial in 2002 (Kasraee, 2002). The mechanism behind this depigmentation is thought to be that methimazole can inhibit tyrosinase, and epidermal melanocytes synthesize melanin pigments by a multistep enzymatic process, initiated by tyrosinase (Kasraee, 2002). Depigmentation of ears of cats has not been reported so far after topical methimazole application.

Methimazole applied topically to treat melasma, at a dose of 5 mg per application, was safe in people, and at this dose caused no thyroid suppression (TSH, fT4 fT3) (Kasraee et al., 2008). A slight tingling of the skin was noticed for a few minutes in five out of nine people (Kasraee et al., 2008). The conclusion from this study was that topical methimazole at 5 mg twice a day was safe and would not cause thyroid suppression. This study cited previous mutagenicity research of methimazole in male mice (Hashimoto et al., 1987), to conclude that: "Present data together with the previously shown non-cytotoxic (Kasraee et al., 2004) and non-mutagenic characteristics of methimazole indicate that this agent could be considered as a safe skin-depigmenting compound for topical treatment of skin hyperpigmentary disorders in humans". The dose of methimazole used in the study by Kasraee (2008), is comparable to the dose of methimazole applied to the ear of cats (Hoffman et al., 2002; Hoffmann et al., 2003; Lecuyer et al., 2006), therefore similar conclusions could be extrapolated.

However, methimazole is also considered teratogenic to the human foetus (Miyamoto et al., 2002; Dechra, 2010; Bayer, 2012), therefore another concern when administering topical methimazole to cats, is the potential for inadvertent absorption through the skin of people in contact with the cat. Precautions, such as wearing gloves or finger cots are

indicated for people when administering transdermal formulations as the drug can also cross human skin. Washing hands with soap is recommended after use of both oral and transdermal formulations. Recommendations when administering methimazole in any form, are that pregnant women need to wear gloves when handling methimazole, and women of child bearing age should wear gloves when cleaning the litterbox of cats treated with methimazole (Dechra, 2010; Bayer, 2012).

Controversy exists as to the teratogenic potential of methimazole and carbimazole, and previous published data is insufficient to draw a definitive conclusion with no prospective controlled studies supporting the teratogenicity of methimazole (Diav-Citrin & Ornoy, 2002). A recent study determined that there was an association between exposure to methimazole/carbimazole in the first trimester and birth defects such as omphalocele and choanal atresia (Diav-Citrin & Ornoy, 2002; Clementi et al., 2010). The link between exposure to methimazole/carbimazole in the first trimester and these birth malformations definitely suggests that these malformations could be part of a specific, even if rare, embryopathy (Diav-Citrin & Ornoy, 2002; Clementi et al., 2010).

While it is obvious that the transdermal formulation of methimazole which remains on the inner pinna should be treated with the same caution as the initial administration, it has not been considered whether the methimazole applied to the inner pinna can pass through the ear and be within the skin of the outer pinna, and further research is required in this area.

Cats also groom themselves, and the ear will be groomed by using a dampened forepaw to clean. It is currently unknown whether these grooming habits contribute to the serum concentrations of methimazole, as grooming or licking has been shown to contribute to

serum drug concentrations of topically applied drug formulations in cattle (Sallovitz et al., 2005; Toutain et al., 2012).

The next section (**Chapter 1, Section 3.0**) will provide background on the structure and function of feline skin, so that the application of drugs to the inner pinna of cats can be better understood and the following section (**Chapter 1, Section 4.0**) will provide an overview of transdermal drug therapy, including drugs other than methimazole that have been applied in a suitable vehicle to the inner pinna in cats.

3.0 OVERVIEW OF THE STRUCTURE AND FUNCTION OF FELINE SKIN

Before undertaking studies in transdermal drug therapy in cats, an understanding of the anatomy and function of feline skin is required. An overview of the structure and function of the skin is necessary to understand transdermal drug penetration and the factors that can affect absorption. Drugs that are absorbed via the skin, have to disrupt the stratum corneum, penetrate the skin and then be absorbed by the blood stream to be distributed to the site of action. Therefore this section will provide an overview of the anatomy of the skin of the cat.

The skin is comprised of the epidermis, dermis and subcutaneous tissue. Each layer of the skin is anatomically and physiologically different Figure 1.3.1. The skin is the largest and most visible organ in the body. One of the functions of the skin is a barrier to the outside environment, preventing penetration and absorption of hydrophilic and ionic compounds. Another function of the skin that can impact transdermal drug delivery, is thermoregulation. The hair for insulation, sweat glands for evaporation and blood perfusion for modulation of heat transfer can all affect the rate of transdermal absorption. Other functions of the skin includes mechanical support, neurosensory reception, an endocrine function (such as Vitamin D metabolism), immune roles (such as the antigen presenting Langerhan's cells of the epidermis) and glandular (such as sweat and sebaceous glands) secretion.

The full thickness skin of cats is thinner than dogs, with an average thickness of 0.4–2.0 mm. Cat skin is thickest in the dorsal regions and proximal limbs, and thinnest on the ventrum and distal limbs (Strickland & Calhoun, 1963). The stratum corneum and epidermis of cat skin is also thinner than dog skin (Monteiro-Riviere et al., 1990), with the abdomen and ear having the thinnest stratum corneum (Table 1.3.1).

Cats have different hair follicles from dogs. Both dogs and cats have compounded hair follicles, however the cat has a single primary follicle surrounded by up to five compound follicles, each with three primary and up to 12 secondary hairs (Strickland & Calhoun, 1963).

Table 1.3.1: Stratum corneum thickness, epidermal thickness, and number of epidermal cell layers at various sites on the dog and the cat as determined in frozen sections. (Mean ± SD) Adapted from (Monteiro-Riviere et al., 1990).

Species	Area	Number of Epidermal cell layers	Epidermis (μm)	Corneum (μm)	Blood flow (ml /min per 100 g)
Cat	Abdomen	2.07 ±0.12	11.12 ± 1.76	8.15 ± 1.29	6.19 ± 0.94
	Back	1.60 ± 0.19	11.58 ± 2.33	9.73 ± 1.01	2.39 ± 0.35
	Buttock	1.60 ± 0.12	10.01 ± 1.77	11.49 ± 2.73	1.82 ± 0.59
	Ear	2.13 ± 0.17	10.01 ± 1.53	8.90 ± 0.91	6.46 ± 2.30
Dog	Abdomen	2.17 ± 0.14	13.75 ± 0.94	12.20 ± 2.12	8.78 ± 1.40
	Back	2.00 ± 0.19	14.21 ± 2.61	11.58 ± 1.83	1.94 ± 0.27
	Buttock	2.11 ± 0.36	17.30 ± 7.52	9.73 ± 1.80	2.21 ± 0.67
	Ear	2.78 ± 0.39	18.53 ± 3.68	15.09 ± 1.83	5.21 ± 1.53
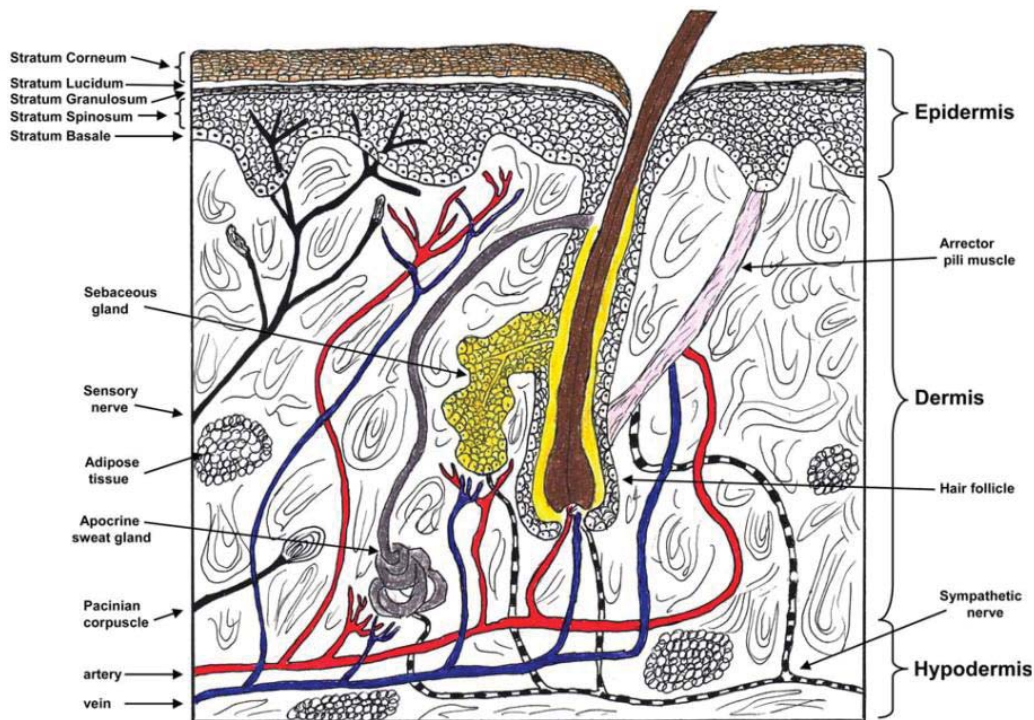
Figure 1.3.1: The typical structure of mammalian skin (from Mills and Cross 2006).

3.1 Epidermis

The outermost skin layer, the epidermis, is the layer responsible for the barrier properties of the skin. The thickness of the epidermis in the cat varies from 10-12 μm depending on the anatomical region (Table 1.3.1).

The epidermis is avascular and composed of four layers; stratum corneum, stratum granulosum, stratum spinosum and the inner most stratum basale at the epidermal-dermal junction (See Figure 1.3.2). A fifth layer, the stratum lucidum, is sometimes described, however this is the lower layer of the stratum corneum. Cells of the epidermis are constantly being renewed, with formation of new keratinocytes at the stratum basale layer. Cells migrating to the outer stratum corneum layer, gradually lose their nucleus and form desiccated proteinaceous corneocytes.

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The stratum corneum regulates water loss and is an efficient physical barrier to chemicals and microbes penetrating the skin. The stratum corneum layer also regulates water loss and disruption to the stratum corneum can result in the penetration of chemicals and drugs. The stratum corneum is the most important barrier for preventing absorption of substances such as drugs (Mills & Cross, 2006b; Benson, 2012). It takes 22 days for a cell from the stratum basale to differentiate into a stratum corneum cell (Paterson, 2008). Typically a cell of the stratum corneum is retained for another 14 days prior to shedding. The cells of the stratum corneum are flattened and consist entirely of keratin. The cells are "cemented" together by an intercellular lipid matrix, which is created by the exteriorized membrane coating granules. This cementing, creates the "bricks and mortar" structure of the stratum corneum. The bricks and mortar analogy is commonly used to illustrate the barrier function of the skin and will be discussed later under drug penetration (**Chapter 1, Section 4.1**).

Disruption to the stratum corneum, can allow chemicals to penetrate through the skin. When the stratum corneum is removed by successive tape stripping with adhesive tape, chemical absorption through the skin will increase (Lademann et al., 2009). Skin surface preparation, with chemicals such as aqueous and alcoholic chlorhexidine, has been shown to disrupt the stratum corneum, resulting in increased penetration of hydrocortisone applied to the skin of the dog in an *in vitro* model (Ahlstrom et al., 2009). Increased hydration of the stratum corneum, due to increased ambient humidity or occlusive dressings, will also increase the absorption of drugs across the skin in pigs (Chang & Riviere, 1993).
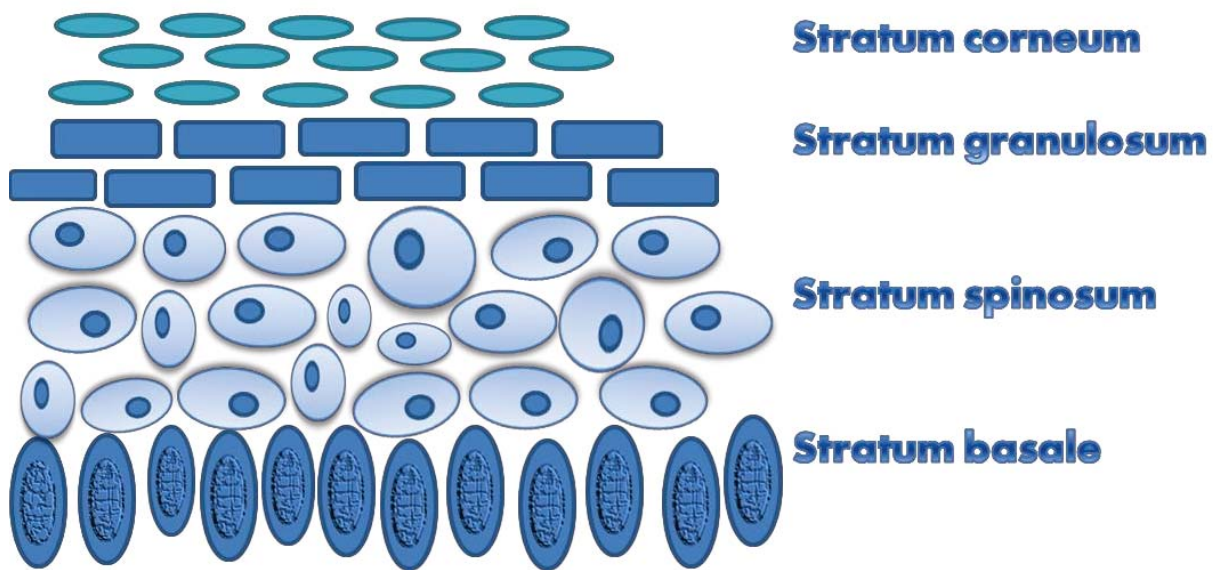
Figure 1.3.2: There are four layers to the epidermis. The stratum corneum is comprised of dead cells and a lipid bilayer. Disruption to this lipid bilayer aids transdermal drug delivery.

3.2 Dermis

The dermis is a vascular layer, which primarily modulates blood flow for thermoregulation. The dermis supplies the avascular epidermis with oxygen and nutrients. The modulation of dermal perfusion can alter transdermal drug delivery, vasoconstriction will decrease dermal delivery and increase local activity, and vasodilation of the dermis can increase systemic delivery of drugs (Riviere & Papich, 2001). The sweat glands and hair follicles in the dermis, may play a role in transdermal drug delivery, which is discussed in the next section, 4.1.

4.0 OVERVIEW OF TRANSDERMAL DRUG DELIVERY

4.1 Penetration of drugs through skin

The skin is a good barrier preventing drugs penetrating the skin surface. The stratum corneum (the non-viable epidermis) is the major layer of the skin that prevents molecules penetrating through to the dermis due to the lipid bilayer (Mills & Cross, 2006b; Benson, 2012). Partition of this lipophilic subcutaneous layer, will allow drugs to move into the more hydrophilic viable epidermis before they reach the systemic circulation.

The absorption of topically applied drugs through the stratum corneum to reach the viable skin layers, deeper tissues or distance tissues via the bloodstream, is a passive process affected by several factors, including the physicochemical properties of both the drug (or permeant) and its vehicle (or formulation that carries the drug) and the physiological conditions of the skin (Mills & Cross, 2006b; Benson, 2012), and involves:

a) the release of the drug from its vehicle;

b) the partitioning of the drug into the stratum corneum;

c) the diffusion of drug through the stratum corneum; and

d) the partitioning of drug from the stratum corneum into the viable epidermis and the dermis.

Drugs for transdermal absorption must be highly lipophilic and formulated in the correct vehicle (Riviere & Papich, 2001).

The following three pathways have been proposed for the absorption of drugs through the barrier provided by the stratum corneum (Figure 1.4.1) (Mills & Cross, 2006b; Benson, 2012):

1. **Intercellular** (via the lipid matrix between the corneocytes)

The intercellular route is the most likely route for drugs to be absorbed through the stratum corneum and is the foundation for the "bricks and mortar" model for solute penetration (Mills & Cross, 2006b). Drugs will move around the keratinocytes through the intercellular lipids which will facilitate the drugs transport.

2. **Transcellular pathway**

Transcellular transport is unlikely and controversial, as drugs would have to go through both the lipophilic intercellular lipids and the hydrophilic corneocytes as well as penetrating the keratinocyte matrix.

3. **Transappendageal**

This pathway is via the skin appendages such as the hair follicles and sweat glands. Transport through hair follicles is controversial, although research has certainly shown that higher concentrations of pesticides will go through skin of the scalp (a skin area with more hair in humans) compared to the skin on the arm (Maibach et al., 1971). The effect of hair follicles could be very important in the penetration of drugs through the skin of cats, due to their higher density of hair follicles compared to humans, and the lipid coating of the hair could also aid drug absorption to the circulation (Mills & Cross, 2006b).

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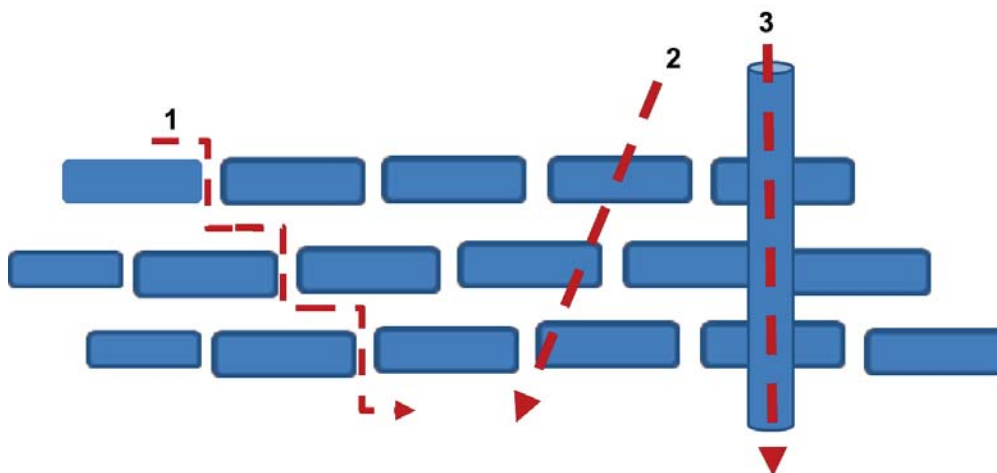
Figure 1.4.1: The possible three pathways for the absorption of drugs through the stratum corneum barrier. 1 = intercellular route, 2 = transcellular, 3 = transappendageal

It is quite likely that drugs may enter the skin by a combination of these pathways, and the exact physicochemical properties of the compound will determine the predominant route. Other skin factors will also affect drug absorption through the skin; age, body site and the prescence of skin pathology all playing a role.

4.2 Drug factors affecting transdermal drug delivery

The penetration of drugs across the skin and particularly the stratum corneum, obey Fick's first law of diffusion :

$$dm/dt = J = DCo\ P/H$$

where dm/dt or J is the steady-state flux, D is the diffusion coefficient of the drug in the stratum corneum, H is the diffusional path length or membrane thickness, P is the partition coefficient between the stratum corneum and the vehicle and Co is the applied drug concentration which is assumed to be constant. Thus the equation helps in identifying the ideal factors influencing the diffusion of a drug across the skin, which are discussed below.

The factors influencing the suitability of a drug for transdermal drug delivery are as follows (Finnin & Morgan, 1999; Chandrasheka & Shobha Rani, 2008):

- potency of the drug - the daily systemic dose should be less than 20 mg per day;

- molecular size - the drug should have a MW of <500 Daltons;

- lipophilicity - the log P should be in the range -1 to 3;

- melting point - should be <200°C;

- hydrogen bonding groups should be ≤2;

- irritation - the drug should not be directly irritant to the skin, as this will increase absorption of the drug; and

- immunogenicity - the drug should not stimulate an immune reaction in the skin.

Carbimazole (Figure 1.1.6) has a molecular weight of 186 Da, a logP 1.35 and a melting point between 122 to 125 °C, methimazole has a molecular weight of 144 Da, logP 0.75 and a melting point of 144 to 147 °C. Both drugs require a daily dose of less than 20 mg per day. Both carbimazole and methimazole are drugs that have properties that should allow good transdermal absorption.

4.3 Vehicle factors affecting transdermal drug delivery

The absorption of drugs through the skin is dependent on the correct vehicle or penetration enhancer to help the drug permeate through the lipid bilayer of the subcutaneous tissue (Barry, 2001; Williams & Barry, 2004). The ideal properties of an enhancer for chemical penetration are listed in Table 1.4.1 (Finnin & Morgan, 1999).

Table 1.4.1: The ideal properties of a chemical penetration enhancer for transdermal drug delivery.

Properties of an Ideal Chemical Penetration Enhancer
Pharmacologically inert
Non irritating, non-allergenic, nontoxic
Non-damaging to viable cells
Rapid onset of effect to allow drug to be absorbed rapidly
Effects of the enhancer are completely and rapidly reversible on removal
Effects do not cause loss of endogenous materials from the body
Physical and chemical compatibility with drugs and excipients in the product applied
Cosmetically acceptable when applied to skin
Odourless, inexpensive, tasteless and colourless

In both the dog and the horse, transdermal penetration of a drug can be greatly influenced by vehicle formulations (Mills et al., 2005; Mills et al., 2006; Ahlstrom et al., 2013).

Some topical formulations are effective due to the occlusion of the stratum corneum, which will increase tissue hydration which permits greater drug influx. Some patches and ointments work in this manner (Riviere & Papich, 2001).

Other penetration enhancers such as pyrrolidones, fatty acids (the most popular being oleic acid) and alcohols (such as ethanol) may alter the lipid bilayer of the skin either by interacting with the polar head groups of the lipids or partitioning of the lipid bilayers of the stratum corneum (Williams & Barry, 2004). Carbimazole and methimazole are mildly lipophilic drugs, which should be combined with the most suitable enhancer to

aid absorption through the skin (Funke et al., 2002; Williams & Barry, 2004 ; Mills et al., 2006; Mills & Cross, 2006b).

4.4 Skin factors for transdermal drug delivery

4.4.1 Blood flow

The vascular upper dermis removes any drugs and solutes that have managed to penetrate through the epidermis. Blood flow to the ear of cats is very good (Table 1.3.1) compared to other regions of the cat. Blood flow in hyperthyroidism is also increased, which may increase the systemic absorption of drugs applied to the skin. It is possible that the blood flow changes that may occur after the cat is euthyroid could explain some of the alterations in methimazole dosing that were required in the long term study of topical administration of methimazole by Boretti et al 2014.

4.4.2 Region of application and individual species differences

The transdermal absorption of drugs can vary greatly depending on the region of application on the body. Studies into the differential regional absorption of transdermal drugs in humans date back to the 1960's, and show that the highest absorption of drugs is from the scrotal area, and lowest in the heel (Feldmann & Maibach, 1967; Moe & Armstrong, 1986; Ebihara et al., 1993). The order of regional variation in skin barrier function in humans is genitals > head and neck > trunk > arm and leg (Feldmann & Maibach, 1967).

Similarly, the region of application has been reported to affect the systemic concentrations of topically-applied drugs in the horse and dog. For example, in the horse, methylsalicylate was absorbed through skin of the leg to a greater extent than the skin of the thorax and groin, while the converse was true for fentanyl (Mills & Cross, 2006a; Mills & Cross, 2007a; Mills & Cross, 2007b). In dogs, hydrocortisone and testosterone in a 50% ethanol vehicle had a nearly two fold greater flux through neck skin compared to inguinal skin (Mills et al., 2005; Mills et al., 2006) and fentanyl was absorbed faster through groin skin than neck skin (Mills et al., 2004b).

In cats, the inner pinna is the most common site for transdermal drug therapy for ointments and gels as opposed to liquid spot-ons and patches (Hoffmann et al., 2003; Marks & Taboada, 2003). The inner pinna is relatively hairless, has a thin stratum corneum and has limited exposure to self grooming (Monteiro-Riviere et al., 1990; Hoffmann et al., 2003).

The differences in skin anatomy between species make extrapolation of transdermal drug absorption kinetics impractical and unwise (Mills & Cross, 2007), and indeed, cats have lower transdermal bioavailability of fentanyl, compared with dogs (Kyles et al., 1996; Lee et al., 2000; Murrell et al., 2007). To date, there are no studies on the regional

application of drugs for percutaneous absorption in cats. Whether the ear is the best site on the skin to apply methimazole for absorption is yet to be confirmed.

4.4.3 Hydration of skin

Hydration of human skin decreases with age (Potts et al., 1984), and as transdermal drug delivery is influenced by tissue hydration, transdermal drug delivery may be altered in older patients. The hydration of the skin in older cats (>10 years) is unknown, however it is possible that aging feline skin is thinner and less elastic. It is possible that older cats with hyperthyroidism may be less hydrated, which could affect the permeability of methimazole across the skin (Anonymous, 2002).

4.5 Drugs for transdermal absorption from the inner pinna previously studied in cats

Not all drugs are suitable for absorption through the skin. This review focuses on drugs absorbed from the pinnal skin of cats. Only a few drugs have been studied in cats to determine whether the drug reaches therapeutic serum concentrations after absorption from the pinna (Scherk Nixon, 1996; Ciribassi et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Bennett et al., 2005; Helms, 2007; MacGregor et al., 2008; Miller et al., 2014; Delamaide Gasper et al., 2015).

The vehicle used for the transdermal drug delivery in cats that has been most commonly studied is Pluronic® lecithin organogel (PLO), with Pluronic® the trade name given to the various polaxmers. PLO was developed in the 1990's by a compounding pharmacist Marty Jones and his collegue Lawson Kloesel (Jones, 2003). PLO is an emulsion of lipid and aqueous substances, that acts like and feels like a gel. The aqueous part of PLO is composed of 20-30% of Pluronic® F-127. The lipid phase of the formula is equal parts lecithin and either isopropyl palmitate or isopropyl myristate (20% by weight or 22% by volume). To prepare the aqueous phase, Pluronic® F-127 is mixed

with ice cold water and magnetically agitated overnight in the refrigerator. The aqueous

phase must remain at low temperatures (< 4 °C) to remain in a liquid state, as at higher

temperatures it becomes a gel (Jones, 2003). The lipid phase is also let stand overnight

to ensure complete dissolution of lecithin. If a preservative is required, then sorbic acid

at 0.2% can be added to both phases. PLO is then combined using shear force such as

the syringe to syringe method or an electronic mortar and pestle (Jones, 2003). Shear

force will result in uniform micelle production (Figure 1.4.2).

To incorporate PLO with the active drug, the drug can be added to the prepared PLO or

depending on the solubility of the drug, it can be added to either the lipid or aqueous

phase, before the two phases are mixed together. A hydrophilic drug can be mixed with

a small quantity of water before mixing with the aqueous phase, and a hydrophobic drug

can be mixed with propylene glycol prior to mixing with the lipid phase.
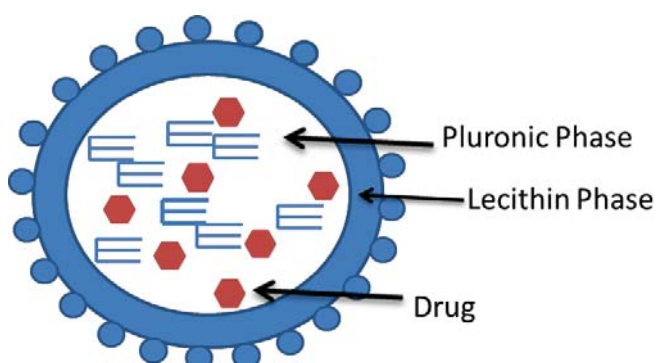
Figure 1.4.2: Micelle of a water soluble drug in Pluronic® Lecithin Organogel.

4.5.1 Evidence for PLO as a vehicle for transdermal drug delivery in cats:

Despite PLO being widely used in veterinary and human medicine as a penetration enhancer, scientific evidence is limited about the physicochemical structure of PLO, such as its stability, rheology, structure, ability to incorporate drugs (Murdan, 2005). A preliminary study into the formulation of methimazole in PLO and Pluronic® gel has been published (Morales et al., 2009). Morales et al (2009), concluded that the vehicles tested were adequate transdermal release systems. A second conclusion from the study was that the vehicles had a large bio-adhesive and absorptive capacity and did not leave residue on the skin.

After the development of PLO gel in 1992, Jones and Kloesel collaborated in the early 1990s with human physicians to treat patients with a variety of drugs incorporated within PLO gel (Jones, 2003). This collaboration resulted in anecdotal evidence of the efficacy of the use of this vehicle in transdermal drug delivery (Jones, 2003). There are still few systematic evaluations of the efficacy of PLO as transdermal drug delivery vehicle in the veterinary and medical literature.

A variety of research methods with varying degrees of evidence have been used to determine the efficacy of drugs absorbed from the pinna of cats. A few studies in cats have measured plasma concentrations after application and whether therapeutic values are reached after transdermal absorption (Scherk Nixon, 1996; Hoffman et al., 2002; Ciribassi et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Bennett et al., 2005; Helms, 2007; MacGregor et al., 2008; Miller et al., 2014; Delamaide Gasper et al., 2015). Other studies used a single application to assess efficacy (Hoffman et al., 2002; Ciribassi et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Bennett et al., 2005) and others multiple applications (Hoffmann et al., 2003; Sartor et al., 2004;

Buijtels et al., 2006; Lecuyer et al., 2006; Helms, 2007; MacGregor et al., 2008; Miller et al., 2014; Delamaide Gasper et al., 2015).

The European Medicine Agency recommends that pharmacokinetics of transdermal medications should be assessed after single and multiple doses, therefore many of these studies in cats may be underestimating bioequivalence compared to oral dosing (Anonymous, 2010). All future transdermal pharmacokinetic studies in cats should include full pharmacokinetic profiles after single and multiple applications.

Clinical efficacy or biological end-points, are a good way of assessing transdermal drug efficacy, as the systemic action of the drug after transdermal application can be monitored. For example, the endpoint of successful treatment of anti-thyroid drugs can be determined by their biological effect, i.e. the decrease in serum TT4 concentrations and resolution of clinical signs rather than the plasma concentration of methimazole alone. Some studies in cats have used clinical parameters or biological endpoints to determine efficacy of transdermal absorption (Hoffmann et al., 2003; Sartor et al., 2004; Bennett et al., 2005; Buijtels et al., 2006; Lecuyer et al., 2006; Helms, 2007; MacGregor et al., 2008).

4.5.2 Evidence for Lipoderm® as a vehicle for transdermal delivery in cats

Lipoderm® contains an alternative to PLO as a vehicle. Lipoderm® is manufactured by Professional Compounding Association of America (PCAA) and according to the manufacturer is superior to PLO. Lipoderm has a smooth and creamy texture and has the advantage that the system is stable and does not separate on refrigeration. Lipoderm® contains a proprietary liposomal component that may increase the permeation of a variety of actives (PCAA). Lipoderm® has been used as the vehicle for amlodipine to successfully treat hypertension in 8 cats (Helms, 2007; Mixon & Helms,

2008). An *in vitro* study performed by the manufacturer demonstrated good penetration through inner feline ear skin of tramadol in Lipoderm®, however a clinical study has yet to be performed (PCAA, 2013). Diltiazem was stable in Lipoderm® for up to 60 days at a concentration of 99.6 mg/mL (Buur et al., 2005). However, when diltiazem in Lipoderm® was assessed in a single dose study in healthy cats relative bioavailability of transdermal compared to intravenous diltiazem was only 10% (DeFrancesco, 2003).

A recently published study of phenobarbital in both PLO (n=7) and Lipoderm® (n=6), applied to the inner pinna of cats for 14 days, showed that therapeutic serum phenobarbital concentrations could be achieved in cats following multiple doses (BID for 14 days) of transdermal administration of phenobarbital in PLO at a dosage of 9 mg/kg q12h, and in most cats at the same dosage in Lipoderm® (Delamaide Gasper et al., 2015). However, there was greater variability seen with the Lipoderm, with some cats having very low serum phenobarbital concentrations (Delamaide Gasper et al., 2015).

A review of studies of drugs that have been applied to the pinna of cats is outlined in Table 1.4.2.

Table 1.4.2: A review of studies of drugs that have been applied to the pinna of cats

Drug	Experimental subjects	Study details	Results
Methimazole **Hoffman et al. 2003**	Hyperthyroid cats n=13	Retrospective study, repeat treatment of methimazole in PLO, no control	Clinical improvement in all cats. Significant difference between pre and post therapy TT4.
Methimazole **Hoffman et al. 2002**	Healthy cats n=6	Triple Cross over study for bioavailability	Low to undetectable concentrations of methimazole. One cat in TD group achieved almost 100% bioavailability compared to oral methimazole route.
Methimazole **Sartor et al. 2004**	Hyperthyroid cats n=27 (TD) N=17 oral methimazole	Prospective randomised clinical trial, repeat treatment methimazole in PLO 4 week duration	Clinical improvement, TT4 decreased and no difference between groups at 4 weeks. Fewer gastrointestinal side effects.
Methimazole **Lecuyer et al. 2006**	Hyperthyroid cats n=13	Prospective trial, repeat treatment methimazole in PLO, no control, 4 week duration	Clinical improvement, TT4 significantly decreased at 4 weeks.
Methimazole **Boretti et al. 2013**	Hyperthyroid cats n=20	Prospective trial, methimazole in PLO gel BID and SID, to determine TT4 suppression	Prolonged TT4 suppression in both BID and SID groups
Methimazole **Boretti et al. 2014**	Hyperthyroid cats n=60	Retrospective trial to assess long term efficacy of methimazole in PLO gel	Median 22 month follow up (4-88mth). Methimazole in PLO is safe long term.
Fluoxetine **Ciribassi et al. 2003**	Healthy cats n=4	Parallel study involving 3 groups of 4 cats to determine biavailability, pharmacokinetics and safety of 5mg/kg and 10mg/kg fluoxetine in PLO, single dose compared to intravenous fluoxetine.	Bioavailability was less than 10% compared to oral dose, fluoxetine TD was absorbed to some extent.
Dexamethasone **Wills-Goulet et al. 2003**	Healthy cats n=5	Cross over study to compare single oral or dexamethasone in PLO	No clinically significant concentrations were detected after TD administration, below quantification limit.

Drug	Experimental subjects	Study details	Results
Amitriptyline and buspirone **Mealey et al. 2004**	Healthy cats n=6	Prospective cross over study, 2 week washout, oral and TD route single dose of amitriptyline (5 mg) and buspirone (2.5 mg) in PLO	Systemic absorption of amitriptyline and buspirone administered by the TD route was poor compared with the per os administration.
Amlodipine **Helms 2007**	6 cats with hypertension	Clinical case series. Cross over study, oral amlodipine followed by Amlodipine in Lipoderm®. Plasma concentrations	Decrease in blood pressure after oral amlodipine by 73 mmHg, increase in blood pressure by 20 mmHg when changed to TD amlodipine.
Phenobarbital **Delamaide et al. 2014**	N=19 Healthy cats.	N=6 PB in PLO was applied to the pinnae for 14 days at a dosage of 3 mg/kg q12h in N=7 PB in PLO at 9 mg/kg q12h, 14 days N=6 PB in Lipoderm Activemax applied at 9 mg/kg q12h for 14 days	Group 1, median concentrations PB ranged from 6.0-7.5 µg/ml Group 2 median concentrations were 26.0 µg/ml (observed range 18.0-37.0 µg/ml). Group 3, median concentrations ranged from 15.0-17.0 µg/ml.
Cyclosporine **Miller et al. 2014**	N=6 Healthy cats	Cyclosporine in PLO controlled, cross-over design, 2 week washout period. Cats were dosed at 5.1–7.4 mg/kg of cyclosporine q 24 hr either per os for 7 days or TD for 21 days	Concentrations of cyclosporine measurable at 7 days after TD therapy, but in therapeutic range in only 1 out of 6 cats.

TD = Transdermal, TT4 = Total Thyroxine concentrations, PLO = Pluronic Lecithin Organogel, PB = Phenobarbital

5.0 TECHNIQUES TO STUDY TRANSDERMAL PENETRATION OF

METHIMAZOLE IN CATS

Drug penetration through the skin can be assessed by *in vitro* or *in vivo* methods.

5.1 Techniques to study transdermal methimazole absorption* in vitro *studies

5.1.1 Diffusion cells

There are several types of diffusion cells available for *in vitro* transdermal penetration

kinetic studies. All of the cells consist of a donor chamber and a receptor chamber

between which the skin is positioned, with the stratum corneum uppermost.

The simplest two-chambered diffusion cell incorporating excised skin, is the Franz –

type diffusion cell (Figure 1.5.1), described by Franz in 1975 (Franz, 1975). Diffusion

cells must be made of inert material such as glass, Teflon or stainless steel, so that if

possible, the test material will not be adsorbed during the experiment.
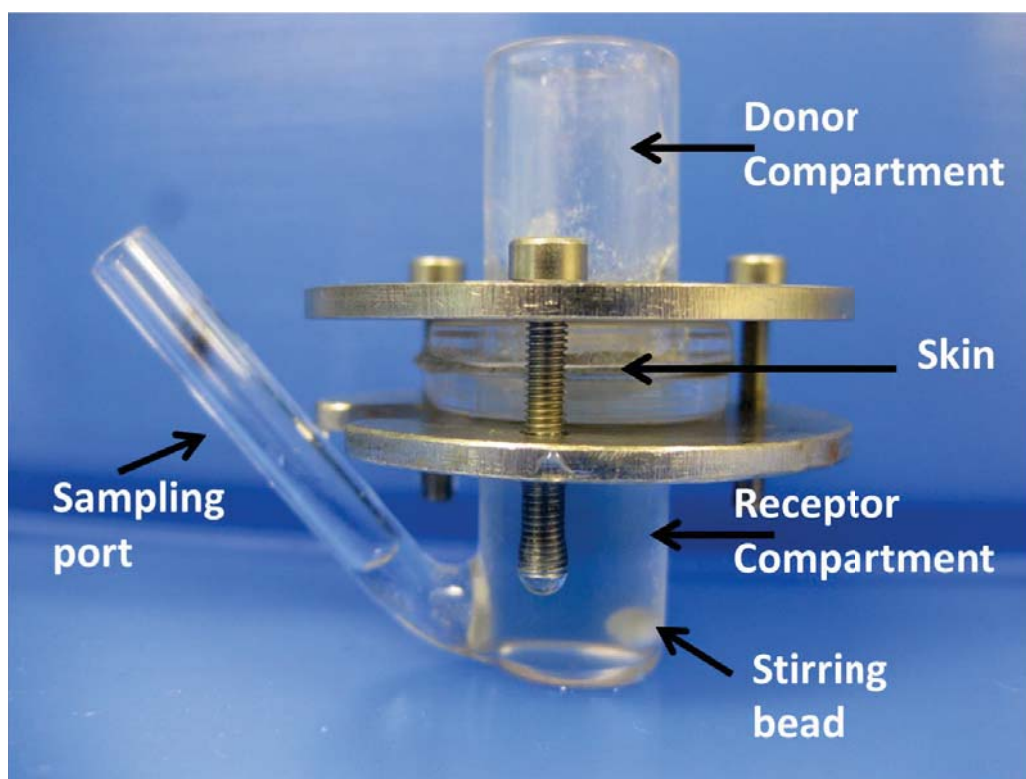
Figure 1.5.1: Photograph of a Franz-type diffusion cell used in **Chapters 5-7.**

The Franz-type cell was developed to study percutaneous absorption under conditions that simulated skin conditions in real life (Franz, 1975). The skin is clamped between the upper donor compartment, which is open to the ambient laboratory conditions and lower receptor compartment. The receptor compartment simulates the physical conditions surrounding the subcutaneous tissues. Inside the receptor compartment, a donor solution in which the drug will dissolve in, such as buffered isotonic saline, comes in contact with the dermal surface of the excised skin. To mimic *in vivo* skin temperatures of 32 °C (for dogs), the cell is submersed in a water bath, thermostatically controlled to 35 °C (Mills et al., 2005). To maintain homogenous temperatures in the receptor compartment, and prevent a layer of higher drug concentration forming under the skin, a Teflon coated magnetic stirring bar is inserted. The stirring bar (or flea) is driven by a magnetic field plate within the water bath (Figure 1.5.2).
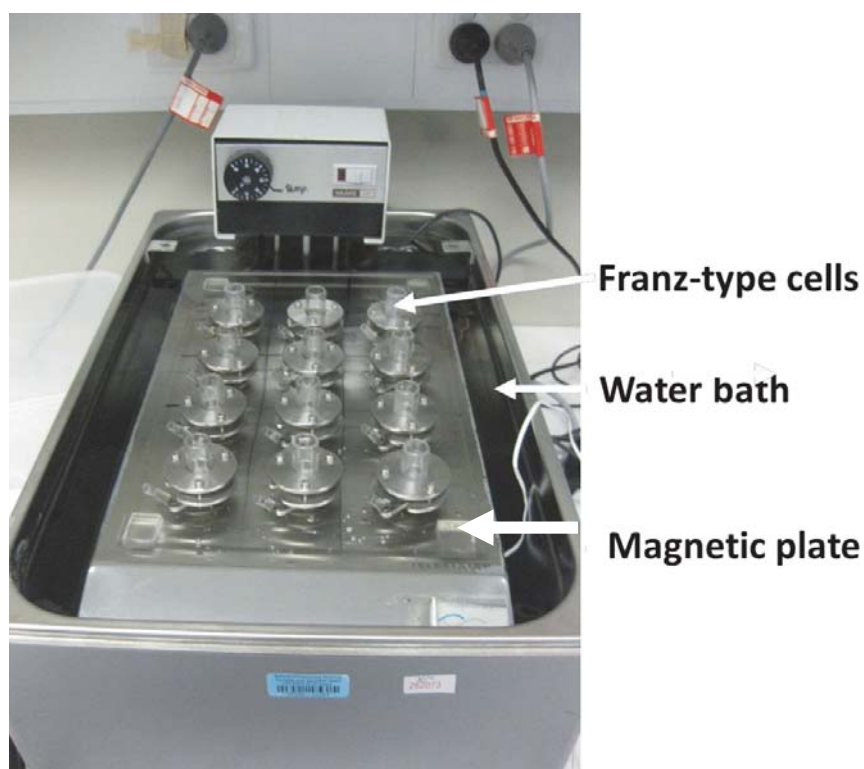
Figure 1.5.2: Franz-type cells in a water bath used in **Chapters 5-7.**

An advantage of the Franz type cell is that a finite (clinically relevant) or infinite (excess) dose of drug can be applied to the stratum corneum within the donor compartment. A finite amount of the drug is used when the test ingredient is replicating a relevant clinical scenario, i.e. a marketed formulation of drug at a set dosage. A finite dose may result in a marked depletion in the donor concentration over the experiment. A finite dose experiment will result in a reduction of the rate of permeation and an eventual plateau effect in the cumulative permeation profile and is discussed later in section 5.1.1c.

5.1.1a Receptor solutions

The receptor solution should be physiologically conducive (i.e. resemble plasma) and must have adequate solubility for the compound being studied, so that the receptor fluid is not a barrier to absorption (OECDa, 2004; Finnin et al., 2012). Adequate sink conditions must be maintained throughout the experiment, which will allow the absorption rate to continue as it would with a functioning circulatory system *in vivo*. Isotonic or buffered saline solutions (pH 7.4) are good for water soluble compounds. Preservatives such as sodium azide can be added to prevent microbial build up if experiments are of long duration or if microbes could contribute to skin metabolism (Finnin et al., 2012).The Organisation for Economic Co-operation and Development (OECD) guidance notes on dermal absorption, state that saline alone may underestimate in vitro absorption for lipophilic compounds. For lipophilic compounds, the receptor fluid should contain solvent mixtures such as ethanol and water (50% aqueous ethanol), <6% polyoxyethelene (20) oleyl ether in water, or 5% bovine serum albumin (OECD, 2011) .

5.1.1b Storage conditions

Storage of skin samples is inevitably required when conducting an _in vitro_ experiment. Studies on canine skin have shown that freezing skin will increase the permeability of hydrocortisone after 1, 4, 8 and 12 months (Ahlstrom et al., 2007). However, the shapes of the permeation profile was similar to fresh skin and no differences were found after histological examination. Freezing of sheep skin found no difference at 1 month, however increased permeability at 6 months (Bayldon, 2012). If skin is not overly hydrated when frozen, it is unlikely that permeation characteristics will be significantly different from those of non-frozen skin (Finnin et al., 2012).

No studies have been performed on frozen cat skin, however it can be is assumed from the above information that short term (< 1 month) freezing of skin would have little effect on permeability, and freezing skin up to 6 months may have a small but predictable effect to increase the permeability of some drugs. The effects of freezing skin can also be assessed by performing histology on the skin samples to determine any freezing artefacts (Ahlstrom et al., 2007).

5.1.1c The permeation experiment

The number of skin replicates recommended in permeation studies is 12 (Finnin et al., 2012). This is due to the high intra and intersubjective variability. If groups are being compared, matched skin samples should be used (Finnin et al., 2012).

The finite dosing experiment

Finite dosing experiments are useful to test a range of clinical drug dose exposures or environmental exposure (Finnin et al., 2012). The finite dose cumulative permeation curve will differ from the infinite curve, as a finite dose will plateau unless a further dose is applied to the membrane surface (Figure 1.5.3). The OECD guideline 428 and

guidance document 28 (OECDa, 2004; OECDb, 2004), define finite dose skin as absorption experiments by the application of ≤ 10 μl/cm² of a liquid formulation to the skin. For semisolid and solid substances, values range between 1 and 10 mg/cm².

The OECD Guideline 428 states for finite dose experiments:

> *When finite dose conditions of exposure are used, the quantity washed from the skin, the quantity associated with the skin (and in the different skin layers if analysed) and the amount present in the receptor fluid (rate, and amount or percentage of applied dose) should be calculated. Skin absorption may sometimes be expressed using receptor fluid data alone. However, when the test substance remains in the skin at the end of the study, it may need to be included in the total amount absorbed (see Guidance Document, paragraph 66).*

Samples of the receptor solution will be taken at regular intervals, most commonly every two hours for 24 up to 48 hours. Early samples (between 1-4 hours) can be important to determine damaged skin permeability (Finnin et al., 2012). A permeation profile (i.e. cumulative amount of drug penetrating per skin area versus time) is plotted.

The parameters that are analysed after the finite permeation study include the flux (*J*) and lag time (t_{lag}). Flux (J) is the amount of drug passing through an area of skin over time (units of mass/area/time). At the completion of the experiment, the membrane or skin can be washed and drug can be extracted from the membrane or skin to calculate the mass balance of drug.

Total recovery of drug is expected to be between 90 to110% (OECDa, 2004). Low recovery rates may be due to the following factors:

- incomplete application of dose

- loss to the experimental equipment

- incomplete extraction from matrices (or incomplete collection of exhaled CO_2)

- evaporation

- unlabelled test preparations, metabolism or degradation

- insufficiently high analytical LODs/LOQs, in particular where non-labelling analytical methods are applied

The Infinite dose experiment

Infinite dose experiments (large excess of permeant that does not deplete over time) are more theoretical experiments as they are not a realistic scenario for most skin exposure to chemicals. However this largely artificial condition is frequently used to determine important kinetic parameters such as flux (J), permeability coefficient (k_P) and diffusion coefficient (D).

The pseudo-steady-state (J_{SS}) can be calculated as the gradient of the regression line through the data points of the linear part of the plotted permeation profile. The J_{SS} represents how much drug moves through the skin in a particular amount of time. By calculating the J_{SS}, and using the known concentration of permeant in the applied formulation (C_V) the permeability coefficient (k_P) can be calculated, using the formula below.

$$\frac{J_{SS}}{C_V} = k_P$$

The permeability coefficient describes how quickly one molecule of the drug moves through the skin and is given in units of cm/h. The lag time ($t$lag) is the initial time taken for the drug to penetrate the skin after the drug is applied, which represents the time taken for a drug to start having effect *in vivo*. Molecules that bind to skin components during permeation will have a longer lag time.

Comparison between the finite and infinite dose experiments

The infinite dose, does not reflect the clinical or occupational exposure scenarios of many drugs or chemicals, however infinite dose exposure can occur such as exposure to chemicals in a swimming pool (Anonymous, 2006). The application of a finite dose best resembles the *in vivo* patient exposure for example the application of an ointment.

The differences between the finite and infinite cumulative absorption profile and flux profile are shown in Figures 1.5.3 and 1.5.3. The cumulative absorption finite curve is expected to plateau as the applied dose of drug to the skin is absorbed. No plateau is seen with the infinite dose, as the excess of applied dose of drug will not deplete and absorption through the membrane/skin can continue. The same is expected for flux (Figure 1.5.4) with the finite dose curve over time being depleted as the drug is absorbed from the membrane of skin.
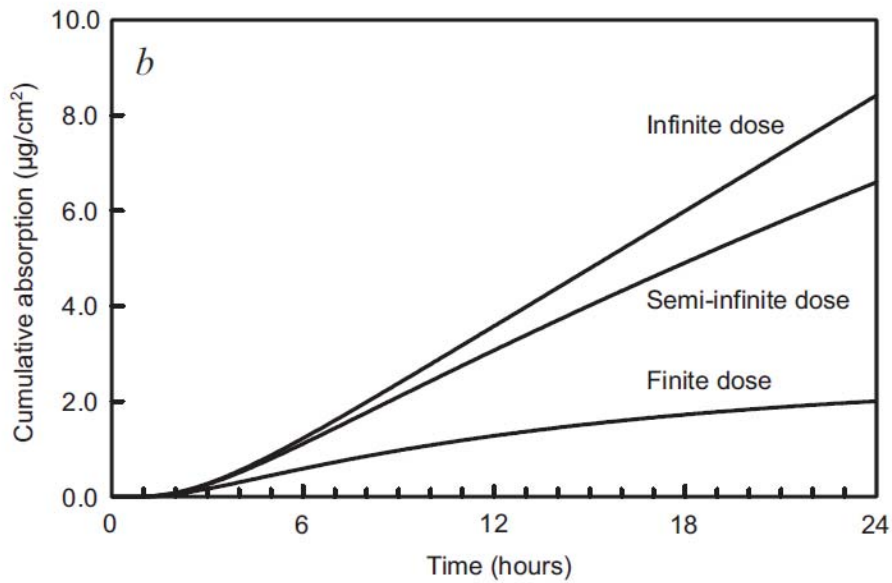
Figure 1.5.3: Simulations of a dermal permeation study: The cumulative amount (μg/cm^2) of drug absorbed through the skin as a function of time in infinite, semi-infinite and finite dose conditions. From Environmental Health Criteria 235: Dermal Absorption (Anonymous, 2006).
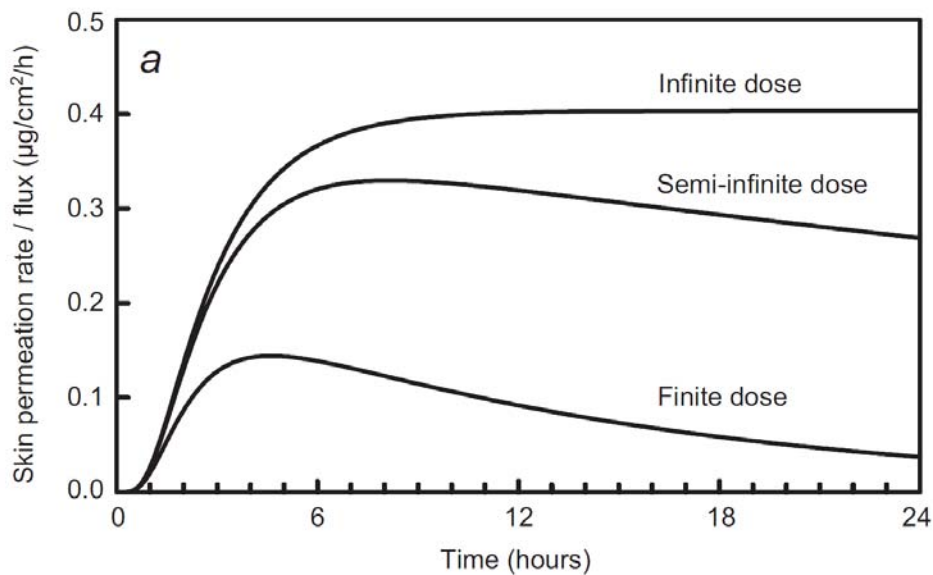
Figure 1.5.4: Simulations of a dermal permeation study: The skin permeation effect of finite and infinite doses of drugs over time for flux (μg/cm^2/h). From Environmental Health Criteria 235: Dermal Absorption (Anonymous, 2006).

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5.2 Techniques to study transdermal methimazole absorption in vivo

5.2.1 Skin sampling

The amount of drug absorbed in the skin in vivo, can be measured by various methods such as tape stripping, multiple full thickness biopsies, shave biopsies and suction blisters. However *in vitro* techniques may be just as useful in providing this data and the ethical cost is lower with *in vitro* techniques (Mills & Cross, 2006b).

5.2.2 Clinical evaluation and Suppression of thyroid function

Carbimazole and methimazole are good drugs to study to determine the systemic availability of the drug after percutaneous absorption as the success of treatment is easily determined by a biological endpoint, i.e. the decrease in serum TT4 concentrations and the resolution of clinical signs (such as increasing body weight) rather than the serum concentration of methimazole alone. Also, objective toxicity data such as monitoring for bone marrow suppression or hepato-toxicity can be performed easily with a CBC and serum biochemistry.

6.0 AIM AND OBJECTIVES OF THESIS

This literature review establishes that hyperthyroidism in cats is a very common disease, and that there are various treatment options for this disease. One of the popular treatment options is medical therapy with drugs such as carbimazole or methimazole. Over the past ten years in countries such as New Zealand, Australia, the USA and most of Europe, methimazole applied to the inner pinna for percutaneous absorption has become an alternative treatment option. However, through this literature review we have seen that there are only a few studies, and most of them clinical case series, to assess the efficacy of methimazole or carbimazole absorption via the percutaneous route. Drugs absorbed from the skin, have particular requirements, and the most commonly used vehicle, PLO gel, might not be the most appropriate vehicle for mildly lipophilic drugs such as carbimazole and methimazole. In addition, whether carbimazole and methimazole are actually absorbed via the skin or are absorbed by the cat self grooming has not been investigated, nor whether the inner ear is the best region of the skin to apply carbimazole or methimazole for transdermal absorption. The deficiencies in the literature have therefore created the aims and the objectives of the research presented in this thesis.

6.1 Thesis aim

The aim of the research studies presented in this thesis was to develop a novel vehicle for the transdermal application of carbimazole/methimazole in cats and to gain further understanding into both the *in vivo* and *in vitro* pharmacokinetics of methimazole in this novel formulation applied to the inner pinna and other skin regions of cats.

6.2 Thesis Objectives

The investigation of the transdermal application of carbimazole/methimazole was described by the following objectives:

1. Develop a novel lipophilic vehicle for the transdermal application of carbimazole/methimazole in cats.

    Hypothesis: Carbimazole/methimazole is more suited to a lipophilic vehicle than PLO gel for transdermal application.

2. Describe the pharmacokinetics of carbimazole/methimazole formulated in a novel lipophilic formulation for application to the inner pinna of cats.

    Hypothesis: Carbimazole/methimazole in a lipophilic vehicle will be absorbed from the inner pinna of cats.

3. Describe the efficacy and safety of methimazole formulated in a novel lipophilic formulation for application to the inner pinnae of client owned hyperthyroid cats.

    Hypothesis: Once daily transdermal administration of a novel lipophilic formulation of methimazole is as safe and effective as oral carbimazole in treating hyperthyroidism in cats.

4. Describe the percutaneous absorption pharmacokinetics of methimazole in a novel lipophilic vehicle compared to the application of methimazole in PLO gel applied to feline ears using a finite dose in an *in vitro* Franz cell model.

    Hypothesis: Methimazole in a novel lipophilic vehicle applied to feline ears will be absorbed to a greater extent than the application of methimazole in PLO gel using a finite dose in an *in vitro* Franz cell model.

5. Compare the absorption of a finite dose of methimazole in a novel lipophilic vehicle formulation through feline skin collected from different regions of the body using an *in vitro* Franz cell model.

   Hypothesis: The inner pinna is the most appropriate site for the absorption of methimazole in a novel lipophilic vehicle.

6. Determination if the percutaneous application of methimazole in a novel lipophilic vehicle formulation, applied to the internal pinna (non-haired region) will cross to the external (haired) pinna of the ear in an *in vitro* Franz type cell model.

   Hypothesis: Methimazole in a novel lipophilic vehicle formulation, applied to the internal pinna (non-haired region) will cross to the external (haired) pinna of the ear in an *in vitro* Franz type cell model.

Two pharmacokinetic studies in healthy cats (one pilot trial), one clinical trial in client owned hyperthyroid cats and three *in vitro* studies using a Franz type cell model were conducted to achieve these objectives.

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Chapter 2

Transdermal administration of carbimazole in healthy cats: a pilot study

PREFACE

As we have seen from **Chapter 1**, both oral carbimazole and methimazole have been used to medically manage cats with hyperthyroidism. **Chapter 1** also discussed that methimazole applied to the inner pinna of cats with hyperthyroidism, would decrease total thyroxine concentrations (TT4), and was a potential alternative therapy for cats with hyperthyroidism. At the time that the research in this chapter was conducted, the only medical therapy to treat hyperthyroid cats in New Zealand was the off label use of carbimazole tablets (5 mg), methimazole was not available, nor the longer acting carbimazole tablets, registered for veterinary use. Therefore the question discussed in this chapter, is whether carbimazole applied to the inner pinna of cats, may also be a suitable therapy for cats with hyperthyroidism.

Prior to a clinical trial of carbimazole applied to the inner ear of cats for the therapy of hyperthyroidism in cats, it is necessary to determine if carbimazole applied to the inner pinna can be absorbed systemically in cats. This chapter reports on a pharmacokinetic pilot trial comparing different formulations of carbimazole applied either to the inner pinna of cats or administered orally. The formulations compared in this chapter are: 1) carbimazole in a vehicle suitable to a lipophilic drug; 2) carbimazole in Pluronic$^{®}$ lecithin organogel (PLO gel); and 3) oral carbimazole. The study was a proof of concept study or pilot study, to determine which vehicle was most suitable to aid the transdermal absorption of the lipophilic drug carbimazole/methimazole. The results of the study were written into a report to secure funding from a New Zealand pharmaceutical company (Bomac Laboratories Ltd, acquisition in 2011 to Bayer NZ Ltd).

INTRODUCTION

As some cats are difficult for owners to medicate with oral drugs, many compounding pharmacies have started formulating drugs into gels. In cats the inner pinna is the most common site for the transdermal application of ointments and gels as opposed to liquid spot-ons and patches. The inner pinna is relatively hairless, has a thin stratum corneum and has limited exposure to self- grooming (Monteiro-Riviere et al., 1990; Hoffmann et al., 2003). In this chapter, transdermal drug therapy refers to drugs applied to the inner pinna of cats.

The aim of transdermal drug delivery is for the drug to enter into the systemic circulation. In contrast, the aim of topical drug therapy is for the drug to remain on local organs. Transdermal drug delivery is a rapidly developing field in both veterinary and human medicine. However, much of the evidence for transdermal drug delivery in cats is anecdotal.

Not all drugs are suitable for transdermal penetration, and only some drugs have been studied to assess the efficacy after transdermal application in cats (Scherk Nixon, 1996; Hoffman et al., 2002; Ciribassi et al., 2003; Hoffmann et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Sartor et al., 2004; Bennett et al., 2005; Buijtels et al., 2006; Lecuyer et al., 2006; Helms, 2007; MacGregor et al., 2008; Miller et al., 2014). A number of studies in cats have determined whether therapeutic serum drug concentrations are reached after transdermal absorption (Scherk Nixon, 1996; Hoffman et al., 2002; Ciribassi et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Bennett et al., 2005; Helms, 2007; MacGregor et al., 2008; Miller et al., 2014). Some studies in cats used a single transdermal application to assess efficacy (Hoffman et al., 2002; Ciribassi et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Bennett et al., 2005), while other studies have used multiple transdermal drug applications

(Hoffmann et al., 2003; Sartor et al., 2004; Buijtels et al., 2006; Lecuyer et al., 2006; Helms, 2007; MacGregor et al., 2008; Miller et al., 2014).

Hyperthyroidism in cats is the most commonly diagnosed endocrinopathy in small animal practice (Mooney, 2010). The use of the human medicines, carbimazole or methimazole/thiamazole to medically manage cats with hyperthyroidism has been standard practice since the discovery of the disease in the early 1980s (Peterson, 1984a; Peterson et al., 1988; Trepanier, 1990; Trepanier et al., 1991a; Mooney et al., 1992; Peterson & Aucoin, 1993; Bucknell, 2000). Carbimazole is a pro-drug of methimazole which is used as tablets to treat hyperthyroid cats in Europe, Australia and New Zealand (Mooney et al., 1992; Bucknell, 2000; Frenais et al., 2008). At the time this study was conducted, the use of carbimazole to treat cats with hyperthyroidism in New Zealand was off-label, as the drug was not registered for veterinary use. In Europe, and now in New Zealand, a once daily long-acting carbimazole tablet (Vidalta®) has been registered for veterinary use, and the FDA recently approved methimazole for the treatment of cats with hyperthyroidism in the USA (Frenais et al., 2008; FDA, 2009; Frenais et al., 2009).

Most compounding pharmacies combine the drug of interest with the vehicle Pluronic® lecithin organogel (PLO) to aid transdermal drug delivery (Boothe, 2006). All previous studies of transdermal methimazole for the therapy of hyperthyroidism in cats have used PLO gel as the vehicle (Hoffman et al., 2002; Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006). Lipophilic drugs (with a log P between -1 to 3) are considered most suitable for transdermal delivery (Finnin & Morgan, 1999; Chandrasheka & Shobha Rani, 2008). Carbimazole (log P 1.35), is a more lipid soluble pro-drug of methimazole (log P 0.75), therefore carbimazole should theoretically be more completely absorbed by the transdermal route than methimazole. A suitable vehicle will allow the active drug to remain soluble and prevent the drug precipitating out of

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solution (Mills & Cross, 2006b). As a vehicle for methimazole, PLO gel has problems

with drug precipitation and also development of a non-homogenous texture to the gel

(Lecuyer et al., 2006). Thus, PLO gel might not be the most suitable vehicle for a lipid

soluble drug (Mills & Cross, 2006b). Carbimazole, has been used as tablets to treat

hyperthyroid cats in New Zealand, and at the time of this trial, carbimazole had not been

studied via the transdermal route. The aim of this pilot study was to develop a lipophilic

vehicle for carbimazole for transdermal administration and to compare this vehicle to

carbimazole in PLO gel and carbimazole tablets per os.

<u>Hypothesis:</u> That carbimazole in the novel lipophilic formulation applied to the inner

ear of cats will reach similar serum concentrations as the clinically proven carbimazole

oral tablets in normal cats.

MATERIALS AND METHODS

Animals

Three healthy research cats and one research cat with mild hyperthyroidism, aged 4 to 8 years of age and weighing 4.0 to 4.5 kg were used. Cats were determined to be healthy based on a complete physical examination and total thyroxine (TT4) concentrations. The cat diagnosed with mild hyperthyroidism, had an increased TT4 (71 nmol/L, ref range 20–40 nmol/L), with no clinical signs of hyperthyroidism (such as vomiting and diarrhoea) and no documented weight loss, therefore was considered eligible for the trial. Cats were provided with water and commercial canned food *ad libitum* and were housed individually during the trial period. All procedures were approved by the Massey University Animal Ethics Committee, Ethics number 05/119.

Formulation of transdermal carbimazole

Two transdermal formulations of carbimazole were prepared: a PLO preparation as previously described (Hoffman et al., 2002) for use with methimazole and a fatty acid/alcohol formulation which may be more suited to a lipid soluble drug. All chemicals were obtained from Sigma Aldrich (St Louis, MO, USA).

Carbimazole in the test lipophilic formulation

To make the test lipophilic transdermal formulation, carbimazole was mixed with the carrier compounds propylene glycol, polyethylene glycol 4000, oleic acid and D limonene. The following proportions were used to make 5.5 ml:

- carbimazole BP 550 mg
- propylene glycol 4 mL
- PEG 4000 1 g
- oleic acid 0.5 mL
- D limonene 0.5 mL

The resulting gel contained 10 mg carbimazole/0.1 mL. Gel was prepared for use within one week of dosing.

Carbimazole in Pluronic® lecithin organogel (PLO)

Lecithin/isopropyl palmitate solution (Solution 1) was prepared using 100 g of granular soya lecithin, 100 g of isopropyl palmitate, and 0.66 g of sorbic acid NF-FCC powder. Pluronic® gel 20% (Solution 2) was prepared with 20 g of Pluronic® F127 NF, 0.3 g of potassium sorbate NF, and purified water, q.s. to 100 mL. Transdermal carbimazole gel was formulated using 0.3 g of carbimazole BP, 0.66 mL of Solution 2 (lecithin/isopropyl palmitate) and enough Solution 2 (Pluronic® F127 gel 20%), to make the solution up to 3.0 mL. The resulting gel contained 10 mg carbimazole /0.1 ml. Gel was prepared for use within one week of dosing.

Experimental protocol

This was a three-period, three-sequence, multiple dose trial with at least a 21 day washout period between treatments (Table 1). The wash out period was based on the following: a) an elimination half-life of methimazole in normal cats ranging from 2–15 h; b) that most drugs are cleared after 4–5 half-lives; and c) that 21 days would provide a seven to eight times safety factor. This length of wash out period should also ensure methimazole concentrations would return to zero and TT4 concentrations would return to the reference range, as well as provide the cats time between blood sampling (Trepanier et al., 1991b).

The two transdermal carbimazole formulations were applied to the inner pinna at dose of 10 mg (0.1 ml) twice daily for 7 days. Oral carbimazole (Neo-Mercazole, AFT Pharmaceuticals, Auckland NZ) was administered at the usual clinical dose of 5 mg twice a day.

Table 1: Treatment groups and blood sampling schedule for three healthy cats and one mildly

hyperthyroid cat given 5 mg of oral carbimazole every 12 h, transdermal carbimazole in a lipophilic

vehicle 10 mg every 12 h and transdermal carbimazole in PLO gel 10 mg every 12 h. * Except on day 7,

only morning dose given. n= number of cats

Group	n	Treatment				
		Day 1–7 Period 1:	Day 8–30 Washout Period 1:	Day 31–38 Period 2:	Day 39–60 Washout Period 2:	Day 61–67 Period 3:
1	4	Oral carbimazole 5 mg q 12 h*	21 days (no treatment)	-	21 days (no treatment)	
2	3	-		Lipophilic Transdermal carbimazole 10 mg q 12 h		
3	4	-		-		Transdermal carbimazole PLO gel 10 mg q 12h

Blood samples

A 20 G catheter was placed in a cephalic vein prior to treatment. Before each blood sample was taken, 2 mL of blood was withdrawn from the catheter. A further 2 mL of blood was then removed for the blood sample. The first 2 mL of withdrawn blood was replaced, followed by 1 mL (10 U heparin/mL) of heparinised saline to prevent occlusion. The samples collected at 0 h and 160 h were 3 mL in volume. Catheters were removed after the 12 h blood sample. Blood samples were collected immediately prior to the start of each treatment, then at 30 minutes, 1, 2, 4, 6, 12 h after the first dose. A final sample was collected via the jugular vein on day 7 (160 h) to estimate the steady state concentrations after multiple dosing. Whole blood samples were allowed to clot for at least 30 minutes. Samples were stored overnight at 4°C. Each sample was centrifuged after coagulation had occurred at 3 000 × g for 5 minutes. The separated serum was immediately frozen in a temperature-monitored freezer at approximately −20 °C until analysis within four weeks.

Serum thyroxine concentrations

Serum thyroxine concentrations were measured at 0 h, 160 h and at the end of the 21 day washout periods, to ensure TT4 concentrations were within reference range prior to commencing another treatment. These samples were analysed at an onsite commercial laboratory, (New Zealand Veterinary Pathology), using a validated commercially available test kit (Immulite, Siemens Los Angeles, CA, USA), with a laboratory reference range of 20–40 nmol/L.

Sample preparation for high performance liquid chromatography (HPLC)

Proteins in the serum samples were removed by precipitation with methanol. Ice-cold methanol (500 µL) was added to 100 µL of serum and the sample vortexed. Samples were incubated on ice for 30 minutes and then centrifuged at 10 000 × g for 5 minutes.

The supernatant was collected and then dried using a Speedvac. The dried samples were dissolved in 100 µL of mobile phase, vortexed and sonicated briefly, and centrifuged at $10\,000 \times g$ for 10 minutes. The supernatant (100 µL) was collected and loaded into the autosampler. All samples were prepared in duplicate for the HPLC.

HPLC Methods

The same HPLC conditions were used for all samples. It was assumed that all

carbimazole was converted to methimazole (Peterson & Aucoin, 1993). Methimazole

standards were run before all samples (Figure 1). Samples were analysed for HPLC as

previously described using a Shimadzu LC20VP system (Trepanier et al., 1991b). The

mobile phase consisted of 0.1 M ammonium acetate, pH 4.0 in 5% (v/v) acetonitrile

made up in MilliQ grade water (Millipore) and filtered with a 0.4 µm filter at a flow rate

of 0.6 mL/minute. The column was a Phenomenex Luna C18, 150 x 4.6 mm, 5 µm, with

a Phenomenex guard column.

For each run, 10 µL was injected, the oven set temperature was 30°C and the detection

wavelength was 254 nm. The run time was 20 minutes. The limit of quantification was

75 ng/mL.
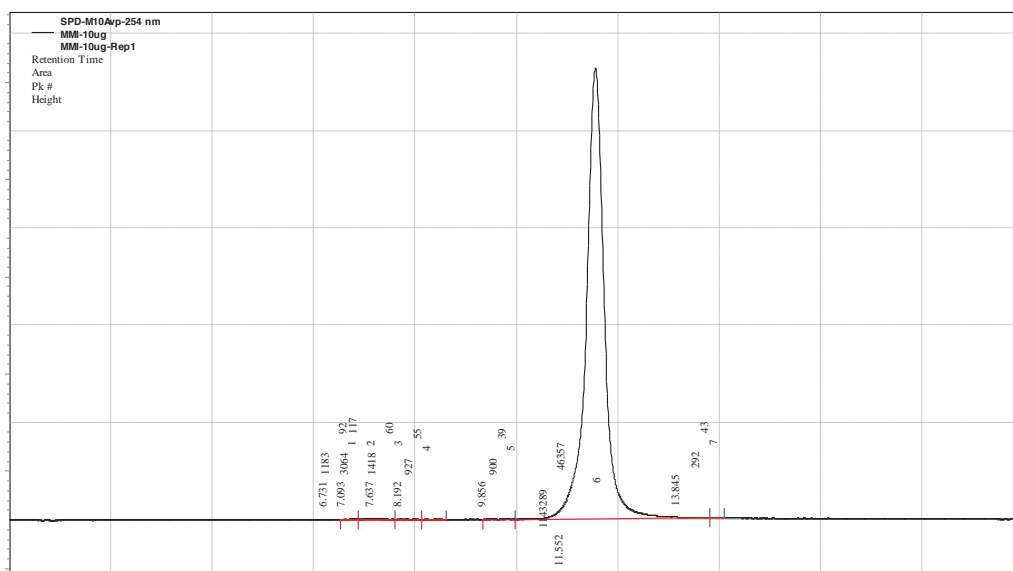
Figure 1: Methimazole (10 µg of methimazole) standard for high performance liquid chromatography

(HPLC) analysis of methimazole from serum. For each run, 10 µL was injected, the oven set temperature

was 30°C and the detection wavelength was 254 nm. The run time was 20 minutes.

Data Analysis for HPLC

Integration of the resulting chromatograms was performed using the Shimadzu VP™ software. Width and threshold were set to 0.5 and 50 respectively. Retention time, area and height were recorded. Standard curves were used to calculate a linear regression curve and the methimazole concentration (µg/mL) in the unknowns calculated from this using Microsoft Excel. For carbimazole in PLO gel, the area and height values were calculated manually as the computer analysis baseline was too low.

Pharmacokinetic analysis

The maximum concentration (C_{max}), time at maximum concentration (t_{max}), elimination half-life ($t_{1/2}$) and area under the curve (AUC) were determined using the PK Solutions software (Summit Research Services, Montrose, CO, USA) using the standard equations.

RESULTS

Four cats were treated with oral carbimazole and carbimazole in PLO gel, however only three cats (one with mild hyperthyroidism) were treated with transdermal carbimazole in the lipophilic vehicle, as one research cat was not available.

The serum concentrations of methimazole following oral and transdermal carbimazole are depicted in Figures 2–4 and the mean serum concentrations in Figure 5.

Oral Carbimazole 5 mg BID
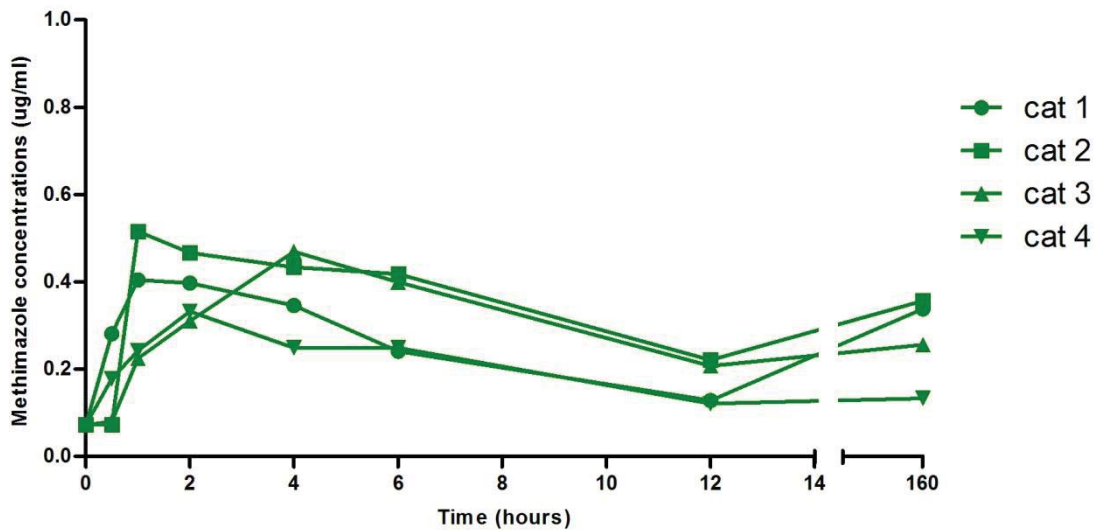
Figure 2: Serum methimazole concentrations following 5 mg oral carbimazole every 12 h (BID) for 14 doses in 4 research cats. Cat 4 had mild hyperthyroidism. Blood samples were collected immediately prior to the start of each treatment, then at 30 minutes, 1, 2, 4, 6, 12 and 160 h after the first dose.

Transdermal Carbimazole lipophilic vehicle 10 mg BID
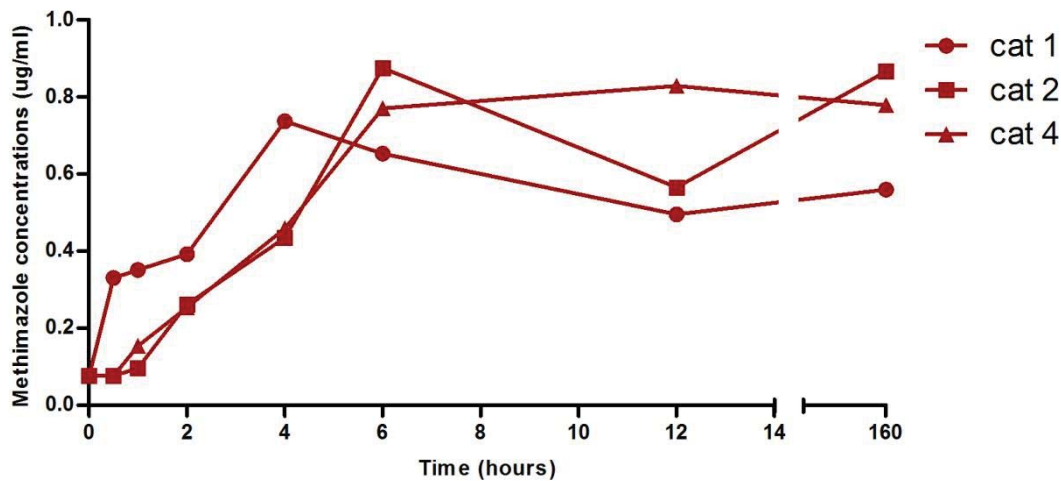
Figure 3: Serum methimazole concentrations following 10 mg transdermal carbimazole in a lipophilic vehicle every 12 h (BID) for 14 doses in 3 research cats. Cat 4 had mild hyperthyroidism. Blood samples were collected immediately prior to the start of each treatment, then at 30 minutes, 1, 2, 4, 6, 12 and 160 h after the first dose.

Transdermal Carbimazole PLO Gel 10 mg BID
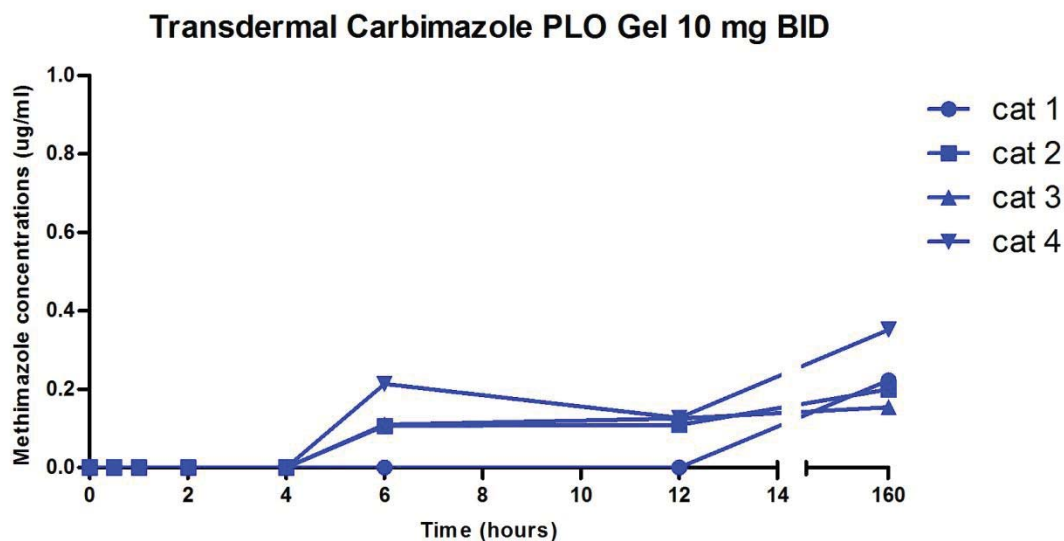
Figure 4: Serum methimazole concentrations following 10 mg transdermal carbimazole in a PLO gel every 12 h (BID) for 14 doses in 4 research cats. Cat 4 had mild hyperthyroidism. Blood samples were collected immediately prior to the start of each treatment, then at 30 min, 1, 2, 4, 6, 12 and 160 h after the first dose.
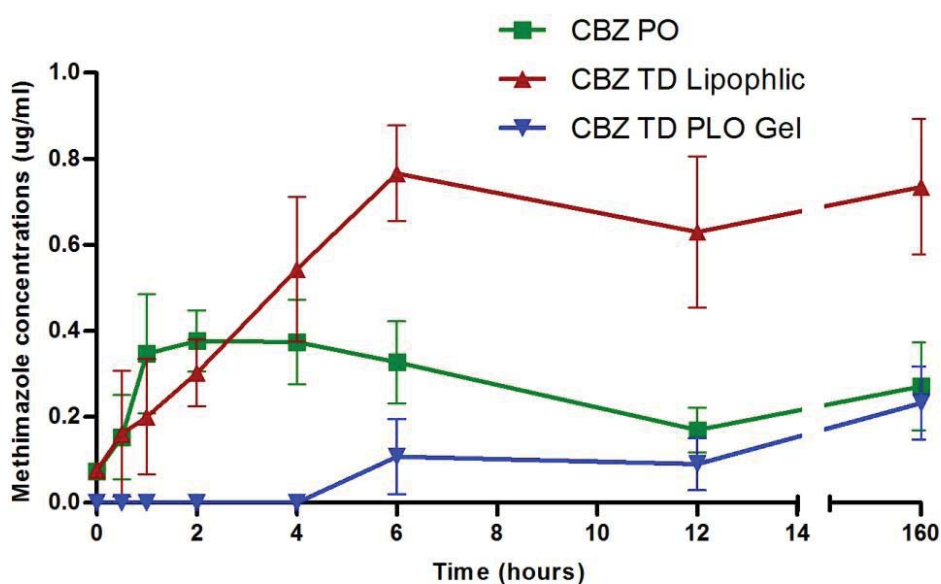
Figure 5: Serum methimazole concentrations (mean, SD) following 5 mg oral carbimazole (CBZ PO) every 12 h (BID) for 14 doses (■) (n=4), 10 mg transdermal carbimazole (CBZ TD) in lipophilic vehicle (▲) (n=3) and 10 mg transdermal carbimazole (CBZ TD) in PLO gel (▼) (n=4) applied every 12 h for 14 doses. Blood samples were collected immediately prior to the start of each treatment, then at 30 minutes, 1, 2, 4, 6, 12 and 160 h after the first dose.

The test lipophilic transdermal formulation, which was administered at double the dose as poor absorption was expected, produced a C_{max} 50% greater than the tablets (0.8 µg/mL SD 0.1, 0.4 µg/mL SD 0.1 respectively), and an AUC almost double (6.7 SD 0.3, 3.5 SD 0.8 respectively) (Table 2). The 7 day serum concentration of methimazole for the test lipophilic transdermal formulation (0.73 µg/mL SD 0.16) was more than double the concentration of oral carbimazole (0.27 µg/mL, SD 0.1) and transdermal carbimazole in PLO gel (0.23 µg/mL SD 0.08).

The carbimazole in PLO gel had non-detectable serum methimazole concentrations until 6 h in 3 out of 4 cats. One cat had non-detectable serum methimazole concentrations through-out the 12 h period, and methimazole concentrations were not detected until 160 h. The serum concentration of methimazole at 160 h was similar to the concentration of methimazole at 160 h after oral carbimazole.

Table 2: Pharmacokinetic analysis based on methimazole concentrations of healthy cats (n=4) treated

with oral carbimazole (5 mg q 12 h) and Transdermal (TD) carbimazole in lipophilic vehicle 10 mg q 12 h

(n=3) and Transdermal carbimazole in PLO gel 10 mg q 12 h (n=4). The pharmacokinetic data through

12 h are based on a single dose. AUC observed

Oral Cabimazole 5 mg

	Cat 1	Cat 2	Cat 3	Cat 4	Mean	SD
C_{max} (μg/mL)	0.4	0.5	0.5	0.3	0.4	0.1
t_{max} (h)	1.0	1.0	4.0	2.0	2	1.4
$t_{½}$ (h)	6.6	6.5	6.3	5.7	6.3	0.4
AUC (μg-hr/mL)	3.1	4.3	3.8	2.6	3.5	0.8
160 h (μg/mL)	0.34	0.36	0.26	0.13	0.27	0.1

Transdermal Carbimazole in lipophilic vehicle 10 mg

	Cat 1	Cat 2	Cat 3	Cat 4	Mean	SD
C_{max} (μg/mL)	0.7	0.9	-	0.8	0.8	0.1
t_{max} (h)	4.0	6.0	-	12	7.3	4.2
$t_{½}$ (h)	15.1	9.5	-	Not reached	12.3	4.0
AUC (ng-hr/mL)	6.6	6.6	-	7.04	6.7	0.3
160 h (μg/mL)	0.56	0.87	-	0.78	0.73	0.16

Transdermal Carbimazole PLO 10 mg

	Cat 1	Cat 2	Cat 3	Cat 4	Mean	SD
C_{max} (ng/mL)		0.1	0.1	0.2	0.1	0.1
t_{max} (h)	Not reached	12	12	6	10.0	3.5
$t_{½}$ (h)						
AUC (ng-hr/mL)	0	0.7550	0.8150	1.237	0.7	0.5
160 h (μg/mL)	0.22	0.20	0.15	0.35	0.23	0.08

AUC = area under the concentration vs. time curve; C_{max} = maximal serum concentration; t_{max}= time at

maximal serum concentration; $t_{½}$ = half-life.

Adverse events

No cats in the oral carbimazole group had any adverse events. Cats in the transdermal carbimazole group showed slight reddening of the application site with both the transdermal preparations after one week of application (Figure 6). No other adverse events were recorded.

Figure 6: Mild erythema and scaling of the ear in a research cat after twice daily application of 10 mg transdermal carbimazole in a lipophilic vehicle applied to the inner pinna for 7 days.

Thyroid hormone concentrations

Table 3 shows the results of the serum TT4 concentrations for cats in the three groups of the study. Cat 4 had mild hyperthyroidism (no clinical signs of hyperthyroidism) with a TT4 (71 nmol/L) concentration well above the reference range (20–40 nmol/L), when not receiving carbimazole therapy, throughout the trial. Thyroxine concentrations returned to pre-treatment ranges after the washout period in all cats. Thyroxine

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concentrations were approximately halved at the end of each 7 day treatment period.

Cat 4 had TT4 concentrations below the reference range after each treatment period.

Table 3: Serum Thyroxine concentrations (reference range 20–40 nmol/L) in cats (n=4) before and after treatment with oral carbimazole 5 mg q 12 h, Transdermal (TD) carbimazole in lipophilic vehicle 10 mg q 12 h and Transdermal carbimazole in PLO gel 10 mg q 12 h.

Animal ID	Thyroxine concentrations (TT$_4$) nmol/l					
	Oral carbimazole		10 mg TD carbimazole lipophilic vehicle		10 mg TD carbimazole PLO gel	
	Day 0	Day 7	Day 28	Day 34	Day 61	Day 67
Cat 1	28	19	30	20	28	20
Cat 2	38	27	25	24	30	25
Cat 3	41	28	N/S	N/S	30	25
Cat 4	71	11	70	11	70	10
Group Mean	44.5	21.3	41.7	18.3	39.5	20.0
SD	18.5	7.9	24.7	6.7	20.4	7.1

[a]cat could not be sampled (N/S)

DISCUSSION

The aim of this pilot trial was to determine whether carbimazole in two transdermal formulations (PLO gel and a novel lipophilic formulation) could be absorbed in the cat, and to compare transdermal absorption with the normal oral route of administration. The lipophilic vehicle appeared to increase the transdermal absorption of carbimazole, as the seven day methimazole concentration in the cats was more than twice the concentration achieved after oral carbimazole and carbimazole in PLO gel applied to the inner pinna. Oral administration of carbimazole produced slightly lower serum levels than published results with methimazole (Trepannier et al., 1991a), but decayed with a similar half- life.

The total daily dose of transdermal carbimazole (20 mg) was double the total daily dose of oral carbimazole (10 mg) as poor absorption was expected with the transdermal formulations. However, the seven day serum concentration of methimazole for the lipophilic vehicle (0.73 µg/mL) was more than double the serum concentration of methimazole in the oral carbimazole group (0.27 µg/mL) and the PLO group (0.23 µg/mL). Therefore it is reasonable to assume, even taking into account the higher dose of carbimazole, that the lipophilic vehicle, does increase the absorption of carbimazole across the pinnal skin. Due to these high serum concentrations of methimazole at seven days, future studies involving the carbimazole in lipophilic vehicle could investigate whether once daily application would be possible.

Carbimazole is a pro-drug of methimazole, with a molecular weight of 186 compared to a molecular weight of 114 for methimazole. Methimazole and carbimazole should be considered equivalent on a molar basis rather than a weight basis, therefore in our study 5mg of carbimazole is equivalent to 3 mg of methimazole, and 10 mg of transdermal carbimazole, equivalent to 6 mg of transdermal methimazole (Jansson et al., 1983).

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In our study, after the first application of 10 mg of carbimazole in a lipophilic vehicle (equivalent to 6 mg methimazole), all three cats had measurable concentrations of methimazole and these concentrations were comparable to the serum concentrations of methimazole after oral administration of 5 mg of methimazole normal cats (Trepanier et al., 1991b; Hoffman et al., 2002). After a single application of transdermal carbimazole in PLO gel (equivalent to 6 mg methimazole) three of four cats had low, but measurable methimazole concentrations at 6 h. There is only one study on methimazole in PLO gel (5 mg dose) applied to the inner pinna of healthy cats (Hoffman et al., 2002). In the study by Hoffman et al., (2002), only two of six cats had measurable methimazole concentrations over a 24 h period. In our study, the serum methimazole concentrations after oral 5 mg carbimazole treatment (equivalent to 3 mg methimazole), were still more than 20% lower than the previously published studies using oral 5 mg methimazole (Trepanier et al., 1991a; Trepanier et al., 1991b).

The starting dose of 10 mg twice daily for transdermal carbimazole was chosen for this study based on published studies of transdermal methimazole (Hoffman et al., 2002; Hoffmann et al., 2003; Lecuyer et al., 2006). The starting dose of 5 mg twice daily for oral carbimazole was based on published studies of hyperthyroid cats requiring carbimazole doses of 10 to 15 mg per day (Mooney et al., 1992; Bucknell, 2000).

The absorption of drugs through the skin is dependent on the correct vehicle or carrier medium (Barry, 2001; Williams & Barry, 2004). The formulation and proportions of the constituents used in the lipophilic vehicle was based on available information for formulation of vehicles for mildly lipophilic drugs (Funke et al., 2002; Williams & Barry, 2004 ; Mills et al., 2006; Mills & Cross, 2006b). Propylene glycol is often used as the main drug solvent for aqueous gels, with terpenes and fatty acids often used as penetration enhancers as they are derived from natural sources and can be used in low

concentrations (Panchagnula et al., 2004; Williams & Barry, 2004). Limonene is a colourless liquid hydrocarbon classified as a cyclic terpene and can be found in the rind of citrus fruits. Limonene has been shown to promote the enhancement of some lipophilic drugs (Okabe et al., 1989; Williams & Barry, 2004). Oleic acid has also been used in transporting drugs and can work synergistically with propylene glycol (Aboofazeli et al., 2002; Williams & Barry, 2004). Further formulation development will be required to determine if the vehicle is stable and has the correct formulation characterization. Formulation characterization includes macroscopic and microscopic appearance, drug uniformity, adding preservatives and rheology and viscosity testing.

The wash out period in this study, was based on the elimination half-life of methimazole in normal cats which ranges from 2–15 h, with an eight times safety factor. This length of wash out period appeared to be adequate as the methimazole concentrations had returned to zero and TT4 concentrations had returned to their reference range (Trepanier et al., 1991b).

The number of cats used in this pilot trial was less than the number of cats (five to ten) used in previous pharmacokinetic studies (Trepanier et al., 1991a; Trepanier et al., 1991b; Lee et al., 2000; Michels et al., 2000; Hoffman et al., 2002). The number of cats used for this trial, was determined by the number of research cats available at the time. It would be recommended for future pharmacokinetic studies to have a minimum of six to eight cats, this would help assess the degree of biological variability between cats.

Cephalic catheters (20 G) were used for this study to minimise costs. The catheters worked relatively well, however did have a tendency to block towards the end of the 12 h and required manipulation of the leg to obtain an adequate sample. Jugular catheters would also be recommended for future multiple blood sample studies.

In conclusion, this pilot study has confirmed that oral carbimazole at 5 mg twice a day produces serum methimazole concentrations in the published therapeutic range, and that 10 mg carbimazole in PLO gel applied to the inner pinna twice a day, in the short term, does not produce measurable methimazole concentrations. The novel lipophilic formulation of 10 mg carbimazole applied to the inner pinna twice a day, results in serum methimazole concentrations comparable to those after oral administration of 5 mg carbimazole twice a day, and will reduce TT4 concentrations. HPLC methods have been established for the quantification of methimazole.

Although further clinical studies on cats with hyperthyroidism will be required before routine use, this study has produced encouraging results and shown that further work investigating carbimazole/methimazole in a lipophilic vehicle for application to the inner pinna of cats is warranted. Extending this pharmacokinetic study with a larger number of cats would be useful to further assess the individual variability of transdermal absorption of carbimazole in a lipophilic vehicle. Further research into the stability of the lipophilic vehicle and drug combination will also be required.

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Chapter 3

The pharmacokinetics of methimazole in a novel lipophilic formulation administered transdermally to healthy cats

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PREFACE

In **Chapter 2** a pilot trial investigated the pharmacokinetics of a novel lipophilic formulation of carbimazole applied to the inner pinna in healthy cats. After the twice daily application of 10 mg carbimazole in the novel lipophilic formulation to the inner pinna, three healthy cats had serum methimazole concentrations comparable to the oral administration of 5 mg carbimazole twice a day. Following the pilot study in **Chapter 2**, funding was obtained from Bomac Laboraties Ltd and a preliminary patent was obtained on the drug in the lipophilic vehicle. The test lipophilic formulation was investigated by Bomac Laboratories Ltd. chemists who showed that carbimazole was immediately converted to methimazole within the vehicle. However, the pilot trial in **Chapter 2**, did prove the concept that methimazole concentrations were measurable in the serum of cats after application of the test lipophilic formulation to the skin of the pinnae. The lipophilic vehicle was further tested to ensure twelve month stability and a few minor changes to the excipients were performed by the chemists at Bomac Ltd. A patent was obtained for New Zealand (Nanjan, K., Chambers, P., Hill, K. E., & Al Alawi, F. (2007, December 20). *552816, Topical formulation*. Intellectual Property of New Zealand) and further research was conducted in healthy (**Chapter 3**) and hyperthyroid cats (**Chapter 4**), before the drug formulation was commercialised with a trade name (Hyper-T Earspot) and released in New Zealand by Bayer NZ Ltd.

Chapter 2 investigated carbimazole in a novel lipophilic vehicle applied to the inner pinna of cats and also established HPLC methods for the detection of methimazole in the serum of cats. The manuscript presented in **Chapter 3** investigates the pharmacokinetics of the patented formulation of methimazole in a novel lipophilic vehicle administered to the inner pinna of healthy cats. In **Chapter 3**, the HPLC methods to detect serum methimazole concentrations in cats were also refined for maximal sensitivity (**Appendix 2**).

This chapter was published in *The New Zealand Veterinary Journal* and is formatted for the style of that journal.

ABSTRACT

Aims: To determine the pharmacokinetics of a novel lipophilic formulation of transdermal methimazole compared to oral carbimazole.

Methods: Six healthy cats received either 5 mg carbimazole orally every 12 hours for 13 treatments and five healthy cats received transdermal (TD) methimazole (5 mg and 10 mg) once daily on the pinna for seven treatments with twenty-one days between treatments. Serum methimazole concentrations over 24 hours and at 148 hours were determined by high performance liquid chromatography (HPLC).

Results: Serum methimazole concentrations for the 5 mg transdermal dose for the first 24 hours were not reliably detected in all cats, while for the 10 mg methimazole TD dose and 5 mg oral doses all cats had detectable serum methimazole concentrations. The maximum concentration (C_{max}) and area under the curve (AUC) was significantly lower for 10 mg TD (108 (SD 25) ng/mL, 2544 (SD 216) mg-hr/ml, respectively) than 5 mg oral carbimazole (355 (SD 113) ng/mL, 31866 (SD 439) ng-hr/ml, respectively). The peak time (t_{max}) and elimination half-life ($t_{1/2}$) were significantly longer for 10 mg TD (5.2 (SD 1.1) hours, 13 (SD 3) hours, respectively) compared to 5 mg oral carbimazole (2.1 (SD 1.6) hours, 5.1 (SD 1.2) hours, respectively). At 148 hours, mean serum methimazole concentrations were similar for carbimazole (255 (SD 28) ng/mL) and 5 mg TD (204 (SD 76) ng/mL), while 10 mg TD was higher (506 (SD 165) ng/mL). The mean relative bioavailability of 10 mg TD compared to oral carbimazole was 48% (minimum 43, maximum 55).

Conclusions: Methimazole applied at a dose of 10 mg to the pinnae once daily in a novel lipophilic formulation has half the relative bioavailablity of oral carbimazole in healthy cats.

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Clinical Relevance: The transdermal absorption of methimazole applied to the skin of the pinnae of healthy cats in a novel vehicle can reach therapeutic concentrations.

Key words *hyperthyroidism, transdermal, methimazole, once daily*

Abbreviations

AUC   Area under the curve

PLO   Pluronic lecithin organogel

INTRODUCTION

Benign hyperplasia of the thyroid is a common condition in cats older than six years of age (Scarlett, 1994; Edinboro, Scott-Moncrieff et al., 2004). There are four treatment options: surgery, radioactive iodine, nutritional therapy with low iodine diets and anti-thyroid drugs such as methimazole or carbimazole (Peterson, 1984; Peterson, Kintzer et al., 1988; Peterson, 2006). Medical management is recommended prior to surgery to stabilise the patient, and is also recommended prior to radioactive iodine to assess renal function (Trepanier, 2007). Many owners elect to use medical management as a long term therapy (Trepanier 2007). Methimazole is commonly used in North America, with carbimazole, a pro-drug which is rapidly converted to methimazole, used in Europe and the Southern hemisphere (Peterson, Kintzer et al., 1988; Mooney, 2002; Frenais, Rosenberg et al., 2009). Methimazole is actively concentrated in the thyroid gland and acts by blocking tri-idothyronine (T3) and thyroxine (T4) synthesis (Jansson, Dahlberg et al., 1983).

Some cats can be difficult to medicate with oral medications; tablets or even liquids and many compounding pharmacies have formulated various drugs into gels which are applied to the inner pinna and are supposed to be absorbed through the skin for systemic action (Riviere & Papich, 2001; Sartor, Trepanier et al., 2004; Lecuyer, Prini et al., 2006). Only a few drugs have been studied in cats to determine if they reach therapeutic serum concentrations after absorption by this route (ScherkNixon, 1996; Ciribassi, Luescher et al., 2003; Willis-Goulet, Schmidt et al., 2003; Mealey, Peck et al., 2004; Helms, 2007; MacGregor, Rush et al., 2008). However, not all drugs are suitable for transdermal penetration as the drug needs to be highly lipophilic and administered in the correct vehicle (Riviere & Papich, 2001; Mills & Cross, 2006). Transdermal delivery of methimazole when formulated in pluronic lecithin organogel (PLO) vehicle has been

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studied extensively in cats. It was absorbed poorly in pharmacokinetic studies but showed clinical efficacy in some cats after repeated applications (Hoffman, Yoder et al., 2002; Hoffmann, Marks et al., 2003; Sartor, Trepanier et al., 2004). However, the PLO formulation used in these studies may not be the most appropriate vehicle for a lipid soluble drug such as methimazole (Mills & Cross, 2006).

The purpose of this study was to determine the pharmacokinetics of two doses of transdermal methimazole (5 mg and 10 mg) formulated in a novel lipophilic formulation applied once daily compared with our standard treatment 5 mg oral carbimazole twice daily.

MATERIALS AND METHODS

Animals

Six healthy male cats 3 to 8 years of age and weighing 3.5 to 4.5 kg were used. Cats were determined to be healthy based on a complete physical examination and total thyroxine (TT4) concentrations within the reference range. Cats were provided with water and commercial canned food *ad libitum* and were housed individually while intravenous catheters were in place. Cats were weighed on days –1, 27 and 55 as an aid to monitoring their health. All procedures were approved by the Massey University Animal Ethics Committee, Ethics number 06/127.

Drug formulations

Carbimazole was obtained as the standard 5 mg tablet (Neomercazole® AFT Pharmaceuticals) for oral administration. Methimazole for transdermal application was formulated by Bomac Laboratories Ltd (Hill, Gieseg et al., 2011) United States patent application number US 2010/0137389 (Nanjan, Al Alawi et al., 2010), into a concentration of 100 mg/mL. The novel transdermal formulation was composed of methimazole, carrier compounds (propylene glycol, polyethylene glycol 4000, dimethyl formamide and cyclodextrin) and a combination of penetration enhancers selected from fatty acids, terpenes, pyrrolidones, a short chain alcohol, glycol ethers, acetins and triglycerides. The methimazole was from a Good Manufacturing Practice certified company. Stability testing, performed under VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products) guidelines for the stability testing of new veterinary drug substances, had determined this product was stable for 12 months after formulation

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(Anonymous, 2007; Nanjan, Al Alawi et al., 2010). The formulation was supplied in 1 mL syringes.

Experimental protocol

This was a three-period, three-sequence, multiple dose trial with a twenty-one day washout period between treatments. The wash out period was based on the elimination half-life of methimazole in normal cats ranging from 2-15 hours with an eight times safety factor. This length of wash out period would ensure methimazole concentrations would return to zero and TT4 concentrations would return to the reference range, as well as to provide the cats time between blood sampling (Trepanier, Peterson et al., 1991a,b).

Cats were sedated with ketamine (10 mg/kg I/V) (Phoenix ketamine injection; Phoenix pharm LTD, Auckland, NZ) and diazepam (0.5 mg/kg I/V) (Ilium diazepam injection; Troy Laboratories, Glendenning, Australia) and a single lumen central venous catheter (Cook, Bloomington, USA) inserted in the saphenous or jugular vein the day before each treatment (days -1, 27 and 55). Catheters were flushed with 1 mL of heparinised saline (10 U heparin/mL) every 8 hours to prevent occlusion. Catheters were removed after final blood samples were taken on days 1, 29 and 57.

Six cats were treated with oral carbimazole, 5 mg every 12 hours for thirteen treatments. After twenty-one days, five of the cats were treated with 5 mg (0.05 mL) transdermal methimazole, applied to the inside of a pinna every 24 hours for seven days. The sixth cat could not be catheterised for blood sampling and was excluded. After another twenty-one days, the five cats were treated in the same manner with transdermal methimazole at a dose rate of 10 mg (0.1mL) every 24 hours for seven days. Transdermal methimazole was administered once daily based on the results of a small

pilot trial (**Chapter 2**) (Hill, Chambers et al., 2006). Gloves were worn for the application of the transdermal methimazole. Cats were individually housed and monitored for the first 4 hours after application. No grooming of the ear was noticed during this time or during the repeated applications.

Blood samples

Blood samples were collected one hour prior to the start of each treatment, then at 30 minutes, 1, 2, 4, 6, 12, 24 and 148 hours after the first dose (Appendix.1) The 148 hour (4 hours after the 7th morning dose) sample was chosen to estimate the steady state concentrations after multiple dosing. Prior to blood sampling, 2 mL of blood was withdrawn from the catheter. A further 1.5 mL of blood was removed for the blood sample. The first 2 mL of withdrawn blood was replaced, followed by 1 mL of heparinised saline. The samples collected at -1 hour and 148 hours were 2.25 mL. Each sample was centrifuged after coagulation had occurred. The separated serum was immediately frozen in a temperature-monitored freezer at approximately –80°C until analysis.

Serum thyroxine concentrations

Serum thyroxine concentrations were measured at -1 hour, 148 hours and at the end of the twenty-one day washout periods, to ensure total thyroxine concentrations were within reference range prior to commencing another treatment. These samples were analysed at an onsite commercial laboratory, (New Zealand Veterinary Pathology), using a commercially available test kit (Immulite, Siemens Los Angeles, USA), with a reference range of 20–40 nmol/L.

Serum methimazole concentrations

The methods for high performance liquid chromatography (HPLC) analysis of methimazole from serum have been previously described (Hill *et al.* 2011) (**Chapter 4**). In summary, samples were analyzed using a Shimadzu LC20VP system in a mobile phase of 0.1 M ammonium acetate, pH 4.0 in 5% (v/v) acetonitrile in water. For each run, 10 µL was injected at a flow rate of 0.6 mL/minute onto the column (Phenomenex Luna C18, 150 x 4.6 mm, 5 micron, with a guard column) at 30°C. The detection wavelength was 252 nm, run time 15 minutes (Appendix 2). Recovery from cat serum was 83% and the limit of quantification was 50 ng/mL and limit of detection 30 ng/ml. Intra-assay variation was ± 3.1% at a 95% confidence level. Inter-assay variation at a 95% confidence level ranged between ± 1.5% and 9.0% over the range of concentrations relevant to the study. Methimazole standards were run before all samples were analyzed.

Pharmacokinetic analysis

The maximum concentration (C_{max}), time at maximum concentration (t_{max}), elimination half-life ($t_{½}$) and area under the curve (AUC) were determined using the PK Solutions software (Summit Research Services, Montrose, CO, USA) using the standard equations. Relative bioavailability (F) for transdermal methimazole was calculated relative to the oral route using the equation, with the daily oral dose being equivalent to 6mg methimazole.

$$F = AUC_{transdermal} \times \text{daily oral methimazole dose}$$

$$/ AUC_{oral} \times \text{daily transdermal methimazole dose}$$

Statistical analysis

Pharmacokinetic parameters were compared using a Welch two-sample t-test (p<0.05 was considered significant). Thyroxine data was tested for normality using the Anderson-Darling test. All pharmacokinetic data was log normally distributed.

Mean serum methimazole concentrations at 148 hours were compared using one-way model (ANOVA) for time, plus the Tukey HSD test for comparing differences between means. For the TT4 concentrations, a linear mixed-effects model, for normally distributed data, was applied to check if there are any differences before or after treatment and between treatments, with the cat treated as a random effect. Statistical analysis was performed using R v 3.0.1 (R Development Core Team, 2013; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Pharmacokinetic analysis

The pharmacokinetic results for healthy cats receiving oral carbimazole 5 mg twice daily and two doses of transdermal methimazole (5 mg and 10 mg once daily) are summarised in Table 1. The concentrations of methimazole in serum following oral and transdermal methimazole are depicted in Figure 1. On a body weight basis, cats received 1.1–1.4 mg/kg of cabimazole orally twice daily. The transdermal dose varied from 1.1–1.4 mg/kg for the 5 mg dose to 2.2–2.8 mg/kg for the 10 kg dose.
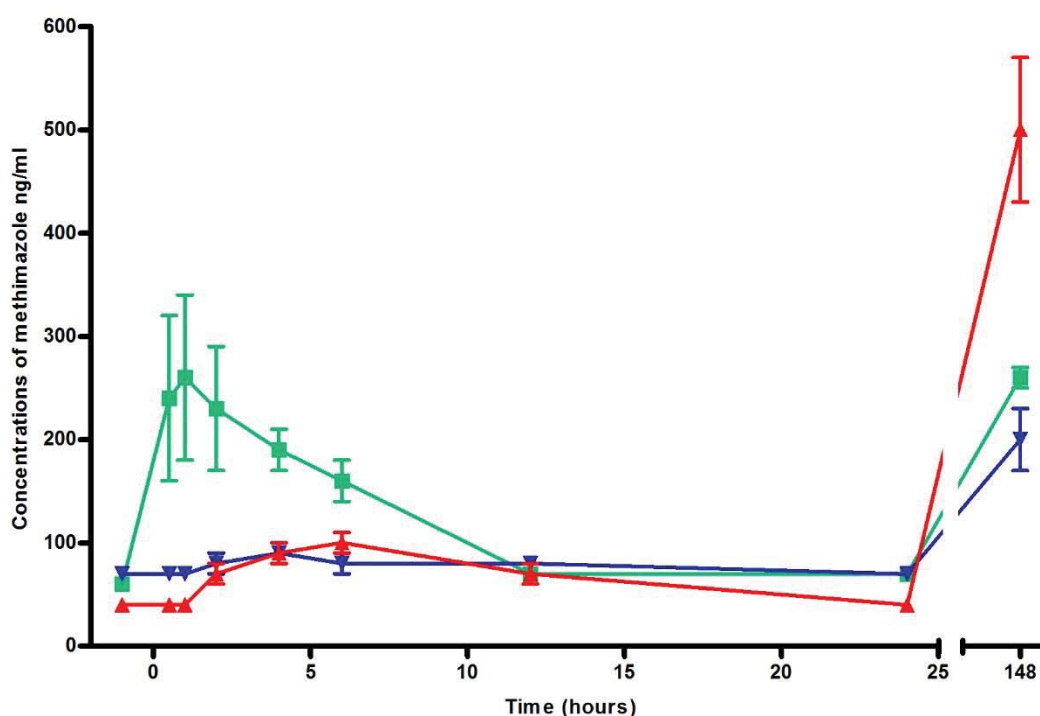
Figure 1. Serum methimazole concentrations (mean, SD) following 5 mg oral carbimazole every 12 hours for 13 doses (■) (n=6), 5 mg transdermal methimazole (n=5) (▲) and 10 mg transdermal methimazole (n=5) (▼) applied every 24 hours for 7 doses. Blood samples were collected one hour prior to the start of each treatment, then at 30 minutes, 1, 2, 4, 6, 12, 24 and 148 hours after the first dose.

All six cats that received 5 mg of oral carbimazole had detectable concentrations of methimazole in serum within the first 12 hours after dosing. After 148 hours of oral carbimazole administration the mean serum concentration of methimazole was 255 ng/mL (SD 28).

Of the five cats treated with 5 mg of transdermal methimazole only two had detectable serum concentrations of methimazole (at two points each) within 24 hours after dosing. Both were only just above the limit of detection. It was decided that the concentrations were too low for further meaningful analysis. All cats in the 5 mg transdermal group had detectable concentrations of methimazole after 148 hours of transdermal treatment with a mean serum concentration of 204 ng/mL (SD 76) that was similar to the oral carbimazole group.

All five cats treated with 10 mg of transdermal methimazole had detectable concentrations of methimazole in their serum within the first 24 hours of dosing. The mean C_{max} (108 ng/mL) and AUC (2544 ng-hr/mL) were both lower than the means for 5 mg carbimazole (p=0.003 and p=0.016 respectively). The t_{max} at 5.2 hours and the elimination half-life at 13 hours were longer than oral dosing of carbimazole (p=0.005 and <0.001 respectively). All cats had detectable concentrations of methimazole after 148 hours and the mean concentration in serum was 506 ng/mL (SD 165), which was higher than the 5 mg carbimazole or 5 mg transdermal methimazole groups (p=0.006 and p=0.002 respectively).

Table 1: Pharmacokinetic analysis based on methimazole concentrations of healthy cats (n=6) treated with oral cabimazole (5 mg q 12 h) and 5 mg and 10 mg of transdermal methimazole once daily. The pharmacokinetic data through 12 hours are based on a single dose.

Oral Carbimazole - 5 mg BID

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Mean	SD
C_{max} (ng/mL)	230	250	510	310	460	370	355	113
t_{max} (h)	4	4	1	2	1	0.5	2.1	1.6
$t_{½}$ (h)	3.4	6.8	4.3	4.5	5.4	6	5.1	1.2
AUC (ng-hr/mL)	2485	3534	3747	3004	3218	3126	3186	439

Transdermal Methimazole - 10 mg

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Mean	SD
C_{max} (ng/mL)	110	150	-	90	90	100	108	25
t_{max} (h)	6	6	-	6	4	4	5.2	1.1
$t_{½}$ (h)	9.5	10.25	-	15.3	16.8	14.1	13	3
AUC (ng-hr/mL)	2435	2674	-	2383	2369	2861	2544	216
F.rel. to p.o. (%)	59%	46%		48%	43%	55%	48%	

Transdermal Methimazole 5mg

	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Mean	SD
C_{max} (ng/mL)	0	110	-	0	110	0	44	60
t_{max} (h)	0	4	-	0	4	0	1.6	2.2
$t_{½}$ (h)	0	51	-	0	40	0	18.2	25.2
AUC (ng-hr/mL)	0	7177	-	0	6554	0	2746	3767

AUC = area under the concentration vs. time curve; C_{max} = maximal serum concentration; t_{max}= time at maximal serum concentration; $t_{½}$ = half life; F = bioavailability relative to oral route.

Adverse events

No cats in the transdermal methimazole group developed pruritus or erythema of the pinnae. No other adverse events were recorded

Thyroid hormone concentrations

Table 2 shows the results for the serum TT4 concentrations for cats in the three groups of the study. There was no significant difference between the TT4 concentrations before and after treatment or between treatments.

Table 2: Serum thyroxine concentrations (reference range 20–40 nmol/L) in cats (n=6) before and after treatment with oral carbimazole 5 mg q 12 h, Transdermal (TD) methimazole 5 mg q 24 h and Transdermal methimazole 10 mg q 24 h.

Animal ID	Thyroxine concentrations (TT$_4$) nmol/l					
	Oral carbimazole		5 mg TD methimazole		10 mg TD methimazole	
	Day 0	Day 6	Day 28	Day 34	Day 56	Day 62
Cat 1	27	19	31	16	26	26
Cat 2	29	27	24	23	32	25
Cat 3	23	28	21	28	24	28
Cat 4	28	34	29	25	28	33
Cat 5	28	26	22	22	25	26
Cat 6	28	25	N/S[a]	N/S	N/S	N/S
Mean	**27.2**	**26.5**	**25.4**	**22.8**	**27**	**27.6**
SD	**2.14**	**4.85**	**4.39**	**4.43**	**3.16**	**3.21**

DISCUSSION

The pharmacokinetic parameters after oral administration of carbimazole, are comparable to previous studies for oral methimazole (C_{max} 1.93 (SD 0.19) µg/mL) and carbimazole (C_{max} 0.8 (SD 0.27) µg/mL) in healthy cats (Trepanier, Peterson et al., 1991a,b; Frenais, Burgaud et al., 2008). Previously, pharmacokinetic studies of transdermal methimazole in PLO gel have shown low bioavailability in healthy cats (Hoffman, Yoder et al., 2002). In this study we found bioavailability of transdermal methimazole at 10 mg to be around 50% when compared to the 5 mg carbimazole oral dose, which is the standard treatment in New Zealand.

The serum $t_{\frac{1}{2}}$ of methimazole when administered at 10 mg transdermally was significantly longer than that after carbimazole was administered orally. The longer $t_{\frac{1}{2}}$ is likely to represent slow dermal absorption of the drug rather than a true terminal $t_{\frac{1}{2}}$ that is due to drug elimination. This "flip-flop" kinetics (Toutain & Bousquet-Melou, 2004) has been reported previously with a slow release oral formulation of carbimazole (Frenais, Burgaud et al., 2008). The rate of elimination of the drug is much greater than the rate of absorption of the drug into the central compartment (plasma) or drug loss from the site of application, resulting in a $t_{\frac{1}{2}}$ value that is not due to elimination.

The mean bioavailability of 10 mg transdermal methimazole relative to twice daily oral carbimazole was 48 (min 43, max 55) % and could be calculated in all 5 cats. A previous study of transdermal methimazole in PLO gel, could determine bioavailability in only 2 of 6 cats (39 and 99%) (Hoffman, Yoder et al., 2002). A limitation of the current study, was not assessing bioequivalence at 148 hours. Future studies on the pharmacokinetics of transdermal medications in cats should assess bioequivalence after single and multiple doses, as suggested by the European Medicine Agency (Anonymous, 2010).

The doses of transdermal methimazole and oral carbimazole were chosen for this study based on previously published literature (Mooney, Thoday et al., 1992; Hoffman, Yoder et al., 2002; Lecuyer, Prini et al., 2006). The doses of transdermal methimazole and oral carbimazole are not equivalent. Carbimazole is a pro-drug of methimazole, with a molecular weight of 186 compared to a molecular weight of 114 for methimazole. Methimazole and carbimazole should be considered equivalent on a molar basis rather than a weight basis, therefore in our study 10 mg daily of carbimazole is equivalent to 6 mg daily of methimazole (Jansson, Dahlberg et al., 1983).

At 148 hours, the 10 mg transdermal methimazole group had a significantly higher mean concentration of methimazole in serum than the oral carbimazole or 5 mg transdermal methimazole groups. The reasons for this higher concentration are unclear, but could include improved bioavailability, higher methimazole dose or drug accumulation over time. We did not study absolute bioavailability, but assumed that carbimazole was fully converted to methimazole. A dermal reservoir of methimazole with slow absorption is likely to avoid the highs and lows of twice daily oral administration and the serum concentrations may thus represent a mean rather than a post-pill nadir. Previously it has been shown that oral methimazole at 5 mg twice daily for 2 weeks did not accumulate (Trepanier, Peterson et al., 1991a,b). However, the serum concentrations of methimazole only show that the drug is absorbed and are not a good representation of the accumulation of methimazole at the level of the thyroid gland, and are unlikely to correlate to the total thyroxine concentrations. Therefore the endpoint for successful therapy of these anti-thyroid drugs is not the serum concentration of drug, but the resolution of clinical signs and the decrease of thyroxine concentrations. This transdermal methimazole formulation has been demonstrated to be effective in treating clinical hyperthyroidism in cats (**Chapter 4**) (Hill et al. 2011).

There was no significant change in concentrations of thyroxine in this study, which was an expected finding. In a previous study of oral methimazole in normal cats, thyroxine concentrations took 2–4 weeks to show a change (Rutland, Nachreiner et al., 2009).

The pharmacokinetic properties of this novel lipophilic vehicle for transdermal methimazole have not been evaluated in hyperthyroid cats. It is quite possible that hyperthyroid cats could be similar to hyperthyroid humans and initially have increased perfusion around the pinnae which might initially affect the pharmacokinetics, however once the hyperthyroidism is controlled, this increase in perfusion would diminish (Weiss, Milman et al., 1993).

Previous studies of methimazole administered orally and intravenously to cats with hyperthyroidism have shown that the elimination half-life (2.3 and 2.5 hours respectively) was shorter compared to healthy cats (4.7 and 5.1 hours) (Trepanier, Peterson et al., 1991a). It is unknown whether this shorter $t_{1/2}$ persists once the hyperthyroidism is resolved but is probably less important when the rate limiting step is absorption of the drug. However, a clinical trial of 20 hyperthyroid cats treated with this novel lipophilic vehicle for transdermal methimazole once daily showed comparable efficacy to 20 hyperthyroid cats treated with oral carbimazole (**Chapter 4**) (Hill, Gieseg et al., 2011).

There are several limitations to this study. An intravenous dose of methimazole or carbimazole was not given, thus the absolute bioavailablity could not be calculated.

The number of cats used in this trial was small, but comparable to similar pharmacokinetic studies in cats (Frenais, Burgaud et al., 2008; Wells, Glerum et al., 2008). Another limitation is that there were no blood samples taken between 24 and 148 hours therefore the pharmacokinetics during this period remain unknown. The site

used for the transdermal application was the pinna, which is the same site that has been used in cats medicated with other transdermal drugs (Hoffman, Yoder et al., 2002; Hoffmann, Marks et al., 2003; Sartor, Trepanier et al., 2004). The site of application might change the rate of drug delivery. A study investigating site variability has been performed in dogs, but not in cats (Mills, Magnusson et al., 2004; Mills, Magnusson et al., 2005; Mills, Magnusson et al., 2006). It is possible that some of the methimazole may have been ingested when the cats in this study were grooming, although the inside of the pinna was chosen to minimise this effect. However, this oral route of absorption will still contribute to the overall pharmacokinetic profile of transdermal drugs (Toutain, Modric et al., 2012).

This study has shown that methimazole in a novel lipophilic vehicle is absorbed transdermally. The pharmacokinetic profile observed supports the administration of 10 mg of methimazole in a novel lipophilic formulation once daily for transdermal absorption to treat cats with hyperthyroidism.

ACKNOWLEDGEMENTS

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Appendix 1

Table 1: Treatment groups and blood sampling schedule for healthy cats (n=6) given 5 mg of oral carbimazole every 12 hours, transdermal methimazole 5 mg every 24 hours and transdermal methimazole 10 mg every 24 hours. * Except on day 6, only morning dose given. n= number of cats.

Group	n	Treatment Day 0–6 Period 1:	Day 7–27 Washout Period 1:	Day 28–34 Period 2:	Day 29–55 Washout Period 2:	Day 56–62 Period 3:
1	6	Oral carbimazole 5 mg q 12 hours*	21 days (no treatment)	-	21 days (no treatment)	
2	5	-		Transdermal methimazole 5 mg q 24 hours		
3	5	-		-		Transdermal methimazole 10 mg q 24 hours

Appendix 2: The methods for high performance liquid chromatography (HPLC) analysis of methimazole from serum were analyzed using a Shimadzu LC20VP system in a mobile phase of 0.1 M ammonium acetate, pH 4.0 in 5% (v/v) acetonitrile in water. For each run, 10 µL was injected at a flow rate of 0.6 mL/minute onto the column (Phenomenex Luna C18, 150 x 4.6 mm, 5 micron, with a guard column) at 30°C. The detection wavelength was 252 nm, run time 15 minutes. Methimazole standards were run before all samples were analyzed, the 40 ng Methimazole (MMI) standard with the peak shown between 7 and 8 minutes is illustrated below.

Chapter 4

The efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism.

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PREFACE

Chapter 3 investigated the pharmacokinetics of a novel lipophilic formulation of methimazole for transdermal absorption across the inner pinna in healthy cats. The study in **Chapter 3** was performed concurrently to the study presented in **Chapter 4.** The study in **Chapter 3** provided evidence that serum concentrations of methimazole were able to be measured in healthy cats following the application of 5 mg and 10 mg of methimazole in a novel lipophilic formulation to the inner pinna of cats for 7 days. The pharmacokinetics of methimazole in the lipophilic formulation supported application to the pinna once a day. However, serum methimazole concentrations do not correlate with whether the formulation will decrease serum thyroxine (TT4) concentrations. Therefore we were interested in the efficacy of this drug formulation in hyperthyroid cats, and whether a topical drug formulation would be a suitable alternative to oral medical therapy for hyperthyroid cats. This chapter investigates the efficacy and safety of a novel lipophilic formulation of methimazole applied to the skin of the pinna once daily for the treatment of cats with hyperthyroidism. At the time this chapter was published (2011), nutritional therapy for hyperthyroidism was not available, and is not referenced in this chapter.

Supplementary information on the posters used to recruit cats for this trial and detailed information of potential adverse reactions that occurred during the trial, which were not included in the published manuscript are included in the appendix section of this chapter.

This chapter was published in *The Journal of Veterinary Internal Medicine* and is formatted for the style of that journal, including the use of US and SI units.

ABSTRACT

Background: Previous studies on transdermal methimazole have used Pluronic Lecithin Organogel as the vehicle. This might not be the most suitable vehicle for a lipophilic drug such as methimazole.

Hypothesis/Objectives: Once daily transdermal administration of a novel lipophilic formulation of methimazole is as safe and effective as oral carbimazole in treating hyperthyroidism in cats.

Animals: 45 client owned cats diagnosed with hyperthyroidism.

Methods: Prospective study. Cats with newly diagnosed, untreated hyperthyroidism were treated with carbimazole (5 mg PO, q 12 h) or methimazole (10 mg) applied to the inner pinnae q 24 h. Cats were examined after 0, 1, 4, 8 and 12 weeks of treatment. Clinical signs and body weight were recorded, and systolic blood pressure, hematological, serum biochemical and urine parameters, total serum thyroxine concentrations (TT4) and serum methimazole concentrations were measured and recorded.

Results: No significant differences between groups were detected at day 0. Both formulations were effective in treating hyperthyroidism. No significant differences were detected in thyroxine concentrations, body weight, blood pressure, heart rate, alkaline phosphatase, alanine aminotransferase, creatinine, urea and urine specific gravity between groups. The serum methimazole concentrations correlated poorly with TT4 concentrations in both groups.

Conclusions and clinical importance: In this 12 week trial, once daily application of a novel formulation of transdermal methimazole applied to the pinnae was as effective and safe as twice daily oral carbimazole in the treatment of cats with hyperthyroidism.

This novel formulation and transdermal application could have practical advantages to some pet owners.

INTRODUCTION

Benign hyperplasia of the thyroid is a very common condition in cats older than six years of age (Scarlett, 1994; Edinboro et al., 2004). There are three treatment options for hyperthyroidism: thyroidectomy, radioactive iodine, or anti-thyroid drugs such as carbimazole (Neomercazole® or Vidalta®) or methimazole (Tapazole®) (Peterson et al., 1988; Peterson & Becker, 1995; Bruyette, 2004; Gunn-Moore, 2005; Peterson, 2006; van Hoek et al., 2007). Radioactive iodine has the fewest adverse effects and is the most efficacious of the treatments, but availability can be limited, upfront costs can be high, clients might not like radiation therapy, and cats with concurrent renal disease are not suitable candidates (Peterson & Becker, 1995; Langston & Reine, 2006; Peterson, 2006; Syme, 2007).

Medical therapy might be an attractive option for clients. Medical management is also recommended prior to surgery to stabilize the patient, and is often recommended prior to radioactive iodine administration to assess renal function (Trepanier, 2007). The use of carbimazole or methimazole to manage cats with hyperthyroidism has been standard practice since the discovery of the disease in the early 1980s (Peterson, 1984; Peterson et al., 1988; Trepanier, 1990; Trepanier et al., 1991b; Mooney et al., 1992; Peterson & Aucoin, 1993; Bucknell, 2000). Carbimazole is a pro-drug of methimazole which is used as tablets to treat hyperthyroid cats in Europe, Australia and New Zealand (Mooney et al., 1992; Bucknell, 2000; Frenais et al., 2008). In New Zealand, the use of carbimazole to treat cats with hyperthyroidism is off-label, as the drug is not registered for veterinary use. In Europe, a once daily long-acting carbimazole tablet (Vidalta®) has been registered for veterinary use, and the FDA recently approved methimazole for the treatment of cats with hyperthyroidism in the United States (Frenais et al., 2008; FDA, 2009; Frenais et al., 2009).

However, some cats are notoriously hard to medicate with oral drugs and medical management of hyperthyroidism which can require twice daily oral tablets. Because of this difficulty, many compounding pharmacies have started formulating drugs into gels, which are applied to the inner surface of cat's ears and are thought to be absorbed through the skin for systemic action (ScherkNixon, 1996; Glerum et al., 2001; Riviere & Papich, 2001; Ciribassi et al., 2003; DeFrancesco, 2003; Hoffmann et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Sartor et al., 2004; Bennett et al., 2005; Boothe, 2006; Buijtels et al., 2006; Lecuyer et al., 2006; Taboada, 2006; Helms, 2007; Plotnick, 2007; MacGregor et al., 2008).

Not all drugs are suitable for transdermal penetration, and only a few drugs have been studied in cats to determine whether they reach therapeutic concentrations within the body after absorption by this route (ScherkNixon, 1996; Ciribassi et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Bennett et al., 2005; Helms, 2007; MacGregor et al., 2008). There are several factors that affect the transdermal delivery of drugs (Mills & Cross, 2006) Firstly the molecule itself needs to be small (< 500 Da), have few atoms available for hydrogen bonding, be lipophilic and have a low melting point. The vehicle must be soluble enough for the drug to dissolve, and the drug itself needs to be able to diffuse through the subcutaneous lipids (Mills & Cross, 2006). For transdermal absorption, the drug must be highly lipophilic and formulated in the correct vehicle (Riviere & Papich, 2001).

All previous studies of transdermal methimazole in cats have used PLO gel as the vehicle, (Hoffman et al., 2002; Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006) however methimazole is a lipophilic drug, and PLO gel might not be the most suitable vehicle for a lipid soluble drug (Mills & Cross, 2006). A suitable vehicle should be soluble enough to contain the active drug without the drug precipitating out of

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solution (Mills & Cross, 2006). As a vehicle for methimazole, PLO gel has problems with drug precipitation and a non-homogenous texture to the gel developing (Lecuyer et al., 2006). Pharmacokinetic studies of methimazole in PLO gel showed that it was poorly absorbed when applied transdermally but did show clinical efficacy in some cats after repeated applications (Hoffman et al., 2002; Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006).

The aim of this study is to compare, in a controlled, prospective clinical trial, the safety and efficacy of oral carbimazole, with a novel lipophilic formulation of transdermal methimazole applied to the pinnae once a day.

MATERIALS AND METHODS

Inclusion criteria

Client-owned cats with newly diagnosed, untreated, naturally occurring hyperthyroidism, that were suitable for medical treatment and had no clinically important other medical disease not attributable to hyperthyroidism, were eligible for the study (see Appendix 1–4). The study was approved by the Massey University Animal Ethics Committee (Ethics number 06/128) and owners gave informed, written consent (Appendix 5). Cats were diagnosed with hyperthyroidism based on a TT4 concentration greater than 3.9 µg/dl (50 nmol/L), measured by chemiluminescence (reference range 1.56–3.12 µg/dl, or 20–40 nmol/L), together with palpable goitre and clinical signs attributable to hyperthyroidism (weight loss, tachycardia, polyphagia, polydipsia or hyperexcitability).

Exclusion criteria

Cats were excluded from entering the trial if an important medical condition was also present that was not attributable to hyperthyroidism. Cats were screened for the trial by one of the authors (KH) after a verbal consultation with the referring veterinarian and a review of the cats history including the results of laboratory tests. Cats were excluded from the trial if owners failed to return for rechecks; facial pruritus developed; clinical signs of illness occurred along with a neutropenia (< 1.0 x 10^9/L); serum alanine aminotransferase (ALT) or alkaline phosphatase (ALP) activity increased to more than twice the week 0 value; persistent clinical signs of vomiting and diarrhoea occurred; or if creatinine concentrations increased above 2.75 mg/dL (250 µmol/L) with clinical signs of illness. Cats were treated with amlodipine[a] (0.625 mg PO/day) for hypertension if the systolic blood pressure was greater than 180–200 mmHg on two consecutive

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visits, or if there was retinal haemorrhage present at the initial visit. Hypertensive cats were excluded from the analysis of blood pressure.

Drug administration

Forty-five cats were enrolled in the study from July 2007 to March 2009. This number was chosen on the basis of a power analysis based on a pilot study. Cats were alternately assigned to receive a starting dose of either oral carbimazole[b] (5 mg q 12 h) or 10 mg (0.1 mL) of the novel formulation of transdermal methimazole applied to the inner pinnae once daily. The transdermal methimazole was applied to the owner's gloved finger and then rubbed onto the non-haired portion of the inner pinnae of alternate ears for each treatment (Appendix 4). The two clinicians involved in the study (KH and DK) were not blinded to the treatment groups.

Methimazole formulation

Methimazole for transdermal application was formulated by Bomac Laboratories Ltd, United States patent number US 2010/0137389 (Nanjan et al., 2010). The novel transdermal formulation was composed of methimazole, carrier compounds (propylene glycol, polyethylene glycol 4000, dimethyl formamide and cyclodextrin) and a combination of penetration enhancers selected from fatty acids, terpenes, pyrrolidones, a short chain alcohol, glycol ethers, acetins and triglycerides. The methimazole was sourced from an approved source from a Good Manufacturing Practice certified company. Stability testing, performed under VICH (International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products) guidelines for the stability testing of new veterinary drug substances, had determined this product was stable for 12 months after formulation (Anonymous, 2007; Nanjan et al., 2010). The formulation was supplied in 1 mL plastic syringes at 100 mg/mL of methimazole.

Monitoring

Cats were re-evaluated at 0, 1, 4, 8 and 12 weeks after the start of treatment. At each evaluation, a physical examination was performed, systolic blood pressure was measured, body weight was recorded and clients were asked to complete a questionnaire about their cat's improvement and tolerance of the drug (vomiting, diarrhoea, appetite, facial pruritus, coat changes, weight loss) (Appendix 1). Blood and urine samples were obtained for a complete blood count[c], serum chemistry[d], urinalysis[e] (UA), TT4 concentrations[f] and methimazole concentrations. Blood samples were collected 6–8 h after a carbimazole dose or 18–20 h after application of the transdermal methimazole formulation. The timing of blood sampling was standardized, but this timing was mainly determined by the availability of the cats and their owners during clinic operational hours. Laboratory testing was performed at an onsite referral laboratory (New Zealand Veterinary Pathology). Methimazole or carbimazole doses were adjusted at weeks 4, 8 and 12 aiming to maintain a TT4 serum concentration within the reference range of 20–40 nmol/L.

High performance liquid chromatography (HPLC) analysis of methimazole from serum

Sample preparation

Whole blood samples from cats were allowed to clot for at least 30 minutes. Blood samples were stored overnight at 4°C and serum was prepared by centrifugation at 3,000 × *g* for 5 minutes and the supernatant collected. Serum was stored at -20°C until use. Proteins in the serum samples were removed by precipitation with methanol. To 100 µL of serum, 500 µL of ice cold methanol was added and the sample vortexed. Samples were incubated on ice for 30 minutes and then centrifuged at 10,000 x *g* for 5 min. The supernatant was collected and then air dried on a heating block at 55°C. The dried samples were dissolved in 100 µL of mobile phase, vortexed and sonicated briefly and centrifuged at 10,000 × *g* for 10 minutes. The supernatant (100 µL) was collected and loaded into the auto sampler. All samples were prepared in duplicate for HPLC.

HPLC Methods

Samples were analysed for HPLC as previously described (Trepanier et al., 1991a). Samples were analyzed using a Shimadzu LC20VP system. The same HPLC conditions were used for all samples. Methimazole standards were run before all samples.

The mobile phase consisted of 0.1 M ammonium acetate, pH 4.0 in 5% (v/v) acetonitrile made up in MilliQ grade water (Millipore) and filtered with a 0.4 µm filter. The column was a Phenomenex Luna C18, 150 x 4.6 mm, 5 µm, with a Phenomenex guard column.

For each run, 10 µL was injected at a flow rate of 0.6 mL/minute, the oven set temperature was 30°C and the detection wavelength was 252 nm. The run time was 15 minutes. The limit of quantification was 75 ng/mL.

Intra-assay variation was ± 3.1% at a 95% confidence level. Inter-assay variation at a 95% confidence level ranged between ± 1.5% and 9.0% over the range of concentrations relevant to the study. Recovery of methimazole from cat serum was calculated to be 83%.

Statistical Analyses

Data were analyzed using linear mixed model methodology that accounted for repeated measures on the same cat and associated correlated errors (nlme Package in R version 2.8.1., The R Foundation for Statistical Computing, Vienna, Austria). The model included the fixed effects of treatment (oral and transdermal), time (week of treatment) and their interaction and the random effect of cat within treatment. Using the Akaike's information criterion, a compound symmetry error structure was determined as the most appropriate residual covariance structure for repeated measures over time within cat. Least squares means and their standard errors were obtained for each treatment for weeks 0, 4, 8, and 12.

RESULTS

Fifty-three cats were screened for this study. Eight cats were ineligible for the trial as they did not meet the inclusion criteria due to other medical diseases not attributable to hyperthyroidism. Forty-five cats with newly diagnosed, naturally occurring hyperthyroidism were enrolled in the study at Massey University Veterinary Teaching Hospital. Twenty-two cats received oral carbimazole at a starting dose of 5 mg q 12 h, and 23 cats received transdermal methimazole at a starting dose of 10 mg once a day. No clients declined enrolment after being assigned to specific treatment groups. Five cats died (three were euthanized) during the trial for reasons unrelated to therapy and were excluded from the statistical analysis. No significant differences between treatment groups were detected at day 0. Both drugs were effective in treating hyperthyroidism as determined by a reduction in TT4 concentrations (Figure 1). Additional evidence of efficacy was demonstrated with an increase in bodyweight, a decrease in blood pressure (Table 1) and improvement in clinical signs (such as decreased appetite) in both groups. Repeated measurements of TT4 concentrations, weight, blood pressure, ALP, ALT, creatinine, urea and USG showed no significant difference between treatment groups. Heart rate was significantly lower ($p=0.05$) and the haematocrit was significantly higher ($p=0.02$) for the transdermal methimazole group compared to the oral group.

Table 1: Selected clinical parameters in hyperthyroid cats treated with oral carbimazole (n = 20) or transdermal methimazole (n = 20) over a 12-week study period. Data are given as least squares means (standard error) obtained from repeated measures analysis.

Variable	Oral					Transdermal				
week of treatment	0	1	4	8	12	0	1	4	8	12
average age (years)	14.0					12.5				
bodyweight (kg)	3.77	3.84	3.89	4.01	4.06	3.75	3.78	3.85	3.88	3.88
(std error)	(0.23)	(0.23)	(0.23)	(0.23)	0.23	(0.23)	(0.23)	(0.23)	(0.23)	(0.24)
HR (beats/min)	207	199	182	185	178	194	184	179	173	170
(std error)	5.9	6.0	5.9	6.0	6.2	6.0	6.9	7.2	7.3	7.3
SAP (mmHg)	172	170	157	160	144	182	172	160	162	158
(std error)	7.3	7.3	7.4	7.3	7.4	7.8	7.9	7.8	7.9	7.8
TT4 µg/dl	9.2	2.2	2.7	2.6	3.2	7.9	2.8	2.4	3.3	2.3
(nmol/L)	(118)	(29.00)	(34.2)	(33.2)	(40.9)	(101.5)	(36.5)	(30.4)	(42.5)	(29.5)
(std error)	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53	0.53
ALT (IU/L)	155	112	70	64	65	145	94	99	104	87
(std error)	16.9	16.9	16.9	16.9	16.9	16.9	16.9	16.9	16.9	16.9
ALP (IU/L)	84	62	35	35	35	83	66	50	50	45
(std error)	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5

Creatinine mg/dl	1.0	1.1	1.3	1.4	1.4	0.9	0.9	1.1	1.2	1.1
(µmol/L)	(95)	(103)	(121)	(127)	(124)	(84)	(87)	(101)	(107)	(104)
(std error)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Urea mg/dl	29.4	30.1	33.2	37.9	35.4	27.5	30.3	32.1	31.5	34.7
(mmol/L)	(10.5)	(10.7)	(11.8)	(13.5)	(12.7)	(9.8)	(10.8)	(11.5)	(11.2)	(12.4)
(std error)	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Hematocrit (L/L)	0.386	0.362	0.357	0.342	0.351	0.412	0.402	0.393	0.383	0.370
(std error)	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012
WBC (x10^9/L)	9.7	10.0	10.3	10.9	9.6	11.5	11.8	10.9	10.7	10.3
(std error)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
USG	1.037	1.039	1.042	1.037	1.040	1.037	1.040	1.041	1.036	1.036
(std error)	0.003	0.003	0.003	0.003	0.004	0.003	0.004	0.004	0.004	0.004

HR – Heart Rate, SAP Systolic arterial Pressure, USG Urine specific gravity
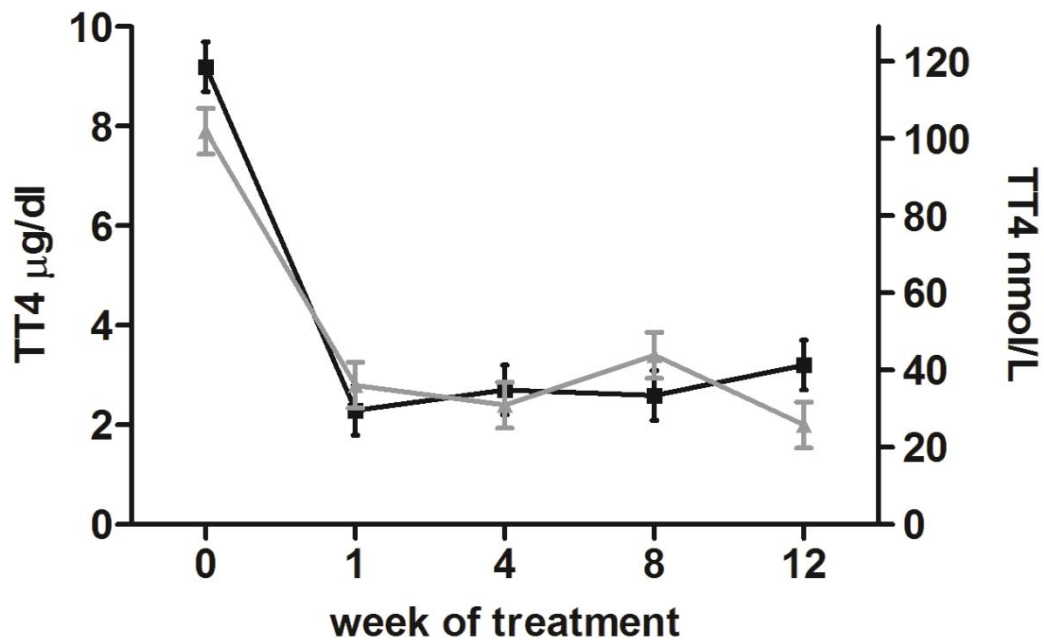
Figure 1: Mean serum total thyroxine (µg/dl and nmol/L) concentrations with standard error bars, in 20 cats treated with oral carbimazole (■) and 20 cats treated with transdermal methimazole (▲).

Methimazole serum concentrations

Methimazole was detected in the serum of cats from both the oral and transdermal groups after administration (Figure 2). However, methimazole concentrations correlated poorly with TT4 concentrations in both groups (Figure 3).
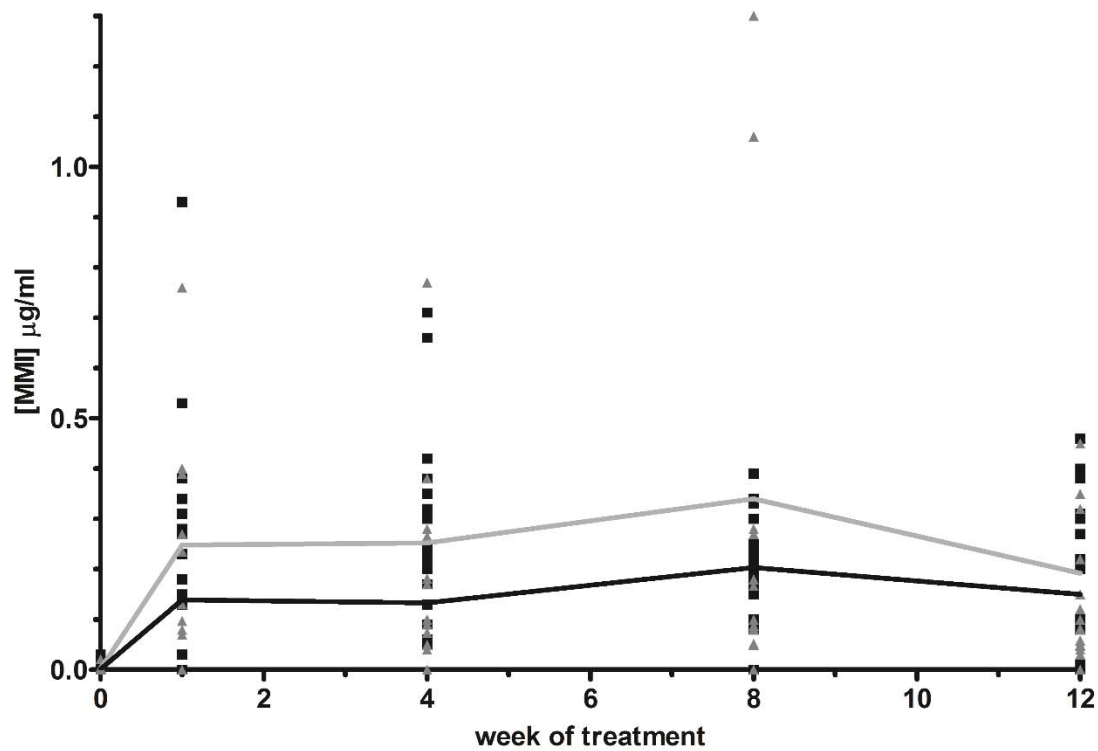
Figure 2: Mean and individual serum methimazole concentrations [MMI] in 20 cats treated with oral carbimazole (■) and 20 cats treated with transdermal methimazole (▲).
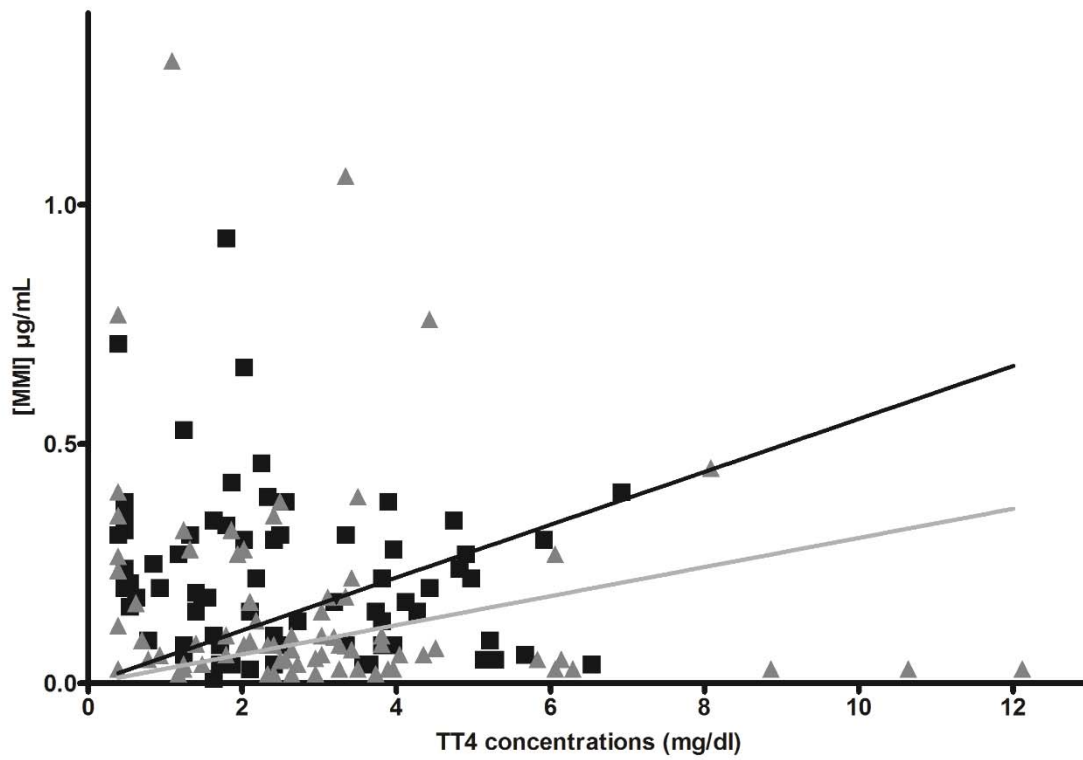
Figure 3: Serum methimazole and serum thyroxine concentrations showing poor correlation between serum methimazole concentrations [MMI] and total thyroxine concentrations (TT4) in cats receiving carbimazole (po) (■) (r^2=0.2) and transdermal methimazole (td) (▲) (r^2=0.16). Data from cats at 4, 8 and 12 weeks of treatment is pooled.

Adverse events

No cats that were enrolled in the trial were removed from the trial based on the exclusion criteria. However, five cats died during the study, but none of the deaths were attributable to the drugs. In the oral carbimazole group, one cat was euthanized 8 weeks after the start of the study due to difficulty eating. A necropsy revealed moderate renal disease; however the exact cause of the difficulty prehending food was undetermined (see Appendix 5). A second cat with a heart murmur, died acutely at week 2. A necropsy was not performed, but acute thrombotic disease was suspected from the owner's description. Two cats in the transdermal group were euthanized (see Appendix 5). The first cat developed pleural effusion and the second cat had hind leg weakness. Necropsies revealed mediastinal lymphosarcoma and iliac thrombus respectively. A third cat in this group was euthanized at 8 weeks in accordance with owner's last will and testament. Six cats (three in each group) developed IRIS (International Renal Interest Society) stage II kidney disease during the treatment trial. One cat in the transdermal methimazole group developed neutropenia at week 4 (day 30) (see appendix 6 for further information). A urine culture revealed no growth of bacteria, and FIV and FeLV tests were negative. The cat was normal and never developed a fever. Transdermal methimazole was stopped for seven days and prophylactic amoxicillin-clavulanic acid[g] (20 mg/kg PO BID) was administered. The cat underwent clinical re-evaluations and a CBC and serum biochemistry at day 33, 37, and 44. At day 37, the cat was demonstrating severe signs of hyperthyroidism (polyuria, polydipsia and polyphagia) and the TT4 concentration was severely elevated (> 15 µg/dL, 193 nmol/L). Transdermal methimazole was reinstated at half the original dose (5 mg once a day) and amoxicillin-clavulanic acid was continued. At day 44, the neutrophil count had returned to normal, antimicrobial therapy was discontinued, and the cat had

no further problems during the trial. A second cat in the transdermal methimazole group developed acute vomiting and anorexia at week 2 of the study. Clinical examination revealed mild abdominal pain and mild elevation in ALT (130 IU/L reference range 0–100 IU/L), abdominal ultrasonography was normal and hepatic fine needle aspirate revealed normal hepatic cells. A clinical diagnosis of acute gastritis was made, and the diet was changed to a low allergenic diet[h]. No changes were made to the transdermal methimazole dose. Within 48 hours the cat was improved and by week 4, the cat was normal with the serum activity of hepatic enzymes decreased (ALT 114 IU/L (reference range 0 - 100 IU/L). One cat in the oral carbimazole group vomited at week 2. Clinical examination was normal and CBC and serum chemistry was normal. The vomiting ceased when the carbimazole was stopped for two days. Vomiting started again when carbimazole was re-introduced at 5 mg twice a day. Vomiting was controlled when carbimazole was decreased to 5 mg every second day in the morning and 5 mg daily at night for 4 weeks (week 4). The carbimazole was then was reinstated at 5 mg twice daily at week 8 with no adverse effects. No cats in the transdermal methimazole group developed pruritus or erythema of the pinnae.

Dose modifications

Eight cats in the transdermal group had dose modifications (Table 4.2). Five cats had a dose reduction and three cats had a dose increase. Thirteen cats in the oral carbimazole had dose modifications, with nine requiring a decrease in the dose and four requiring an increase (Table 4.3). One cat in the transdermal methimazole group never achieved a TT4 concentration (range 4.0–10.7 µg/dL, 51–137 nmol/L) within the reference range, and clinical signs of hyperthyroidism were never completely controlled, although an improvement was noticed. The owner was repeatedly shown how to apply the ointment and questioned over compliance. The owners stated they were applying the ointment

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daily, although the investigators suspected they were not. When the trial was completed

the owners were offered a change to oral carbimazole or radioactive iodine and the cat

responded to radioactive iodine treatment.

Table 2: Dose of transdermal methimazole administered to cats in the study on the efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism.

Daily dosage	Number of cats (percent)			
Dose	**Week 1**	**Week 4**	**Week 8**	**Week 12**
15 mg	0	0	1 (5)	3 (15)
10 mg	20 (100)	18 (90)	14 (70)	12 (60)
5 mg	0	2 (10)	4 (20)	4 (20)
3 mg	0	0	1 (5)	1 (5)
N	20	20	20	20
Median dose (mg)	10	10	10	10
Mean dose (mg)	10	9.5	8.9	9.4

Table 3: Dose of oral carbimazole administered to cats in the study on the efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism.

Dose	Number of cats (percent)			
	Week 1	Week 4	Week 8	Week 12
7.5 mg & 5 mg q12 h	0	0	3 (15)	1 (15)
5 mg q12 h	20 (100)	17 (90)	10 (50)	10 (50)
5 mg q24 h am & 5 mg qod pm	0	1 (5)	0	0
5 mg & 2.5 mg q12 h	0	1 (5)	1 (5)	2 (10)
2.5 mg q12 h	0	1 (5)	6 (30)	6 (30)
2.5 mg & 1.25 mg q12 h	0	0	0	1 (5)
n	20	20	20	20
Median dose (mg)	10	10	10	10
Mean dose (mg)	10	9.6	9.1	8.1

Owner compliance and satisfaction

According to the questions asked of owners at each visit, all owners noticed a clinical improvement in the cats after treatment was instigated. Administering pills to cats in the oral carbimazole group proved to be a challenge with 7/20 (35%) owners admitting to missing doses or cats spitting out the tablet. Owners also reported cats becoming fractious when administering the pills. In the transdermal group, 100% of owners emphasised the ease of application. Two owners reported missing a dose when the cat failed to return home when treatment was due.

Long term follow-up

Fourteen owners of the cats in the transdermal methimazole group elected to continue treating their cats with the transdermal methimazole. A total of 54 TT4 concentrations were measured in the 14 cats over a median follow-up period of 9 months (range 6–24 months). The median TT4 concentration was 2 µg/dL (26 nmol/L) and mean was 2.5 µg/dL (32 nmol/L) (range 0.5-10.5 µg/dL, 6 – 135 nmol/L). No adverse events were recorded.

DISCUSSION

The results of this prospective study show that once daily dosing of transdermal methimazole in a novel lipophilic vehicle is safe and effective in the treatment of spontaneous hyperthyroidism in cats. Furthermore, in the 12 week trial, once daily transdermal methimazole was as effective as twice daily oral carbimazole. Owner compliance was higher in the group treated with once daily transdermal methimazole. The transdermal application was found to have substantial advantages over oral medication as cats tolerated the transdermal medication better than pills. Furthermore, the transdermal preparation required only once daily dosing to achieve disease control. These factors increased owner compliance and lead to effective management of the hyperthyroidism.

Medical therapy is one of the three treatment options available for feline hyperthyroidism (Peterson et al., 1988; Peterson & Becker, 1995; Bruyette, 2004; Gunn-Moore, 2005; Peterson, 2006; van Hoek et al., 2007). The transdermal application of drugs has become popular in feline medicine, as cats can be difficult to consistently medicate orally (ScherkNixon, 1996; Glerum et al., 2001; Riviere & Papich, 2001; Ciribassi et al., 2003; DeFrancesco, 2003; Hoffmann et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Sartor et al., 2004; Bennett et al., 2005; Boothe, 2006; Buijtels et al., 2006; Lecuyer et al., 2006; Taboada, 2006; Helms, 2007; Plotnick, 2007; MacGregor et al., 2008). The vehicle carrier affects the absorption of drugs across the skin, by having a primary role in determining the partition co-efficient for the drug and can also alter the properties of the skin (Riviere & Papich, 2001). The correct vehicle is required for each drug to allow maximum transdermal penetration. As methimazole is a lipophilic drug, a lipophilic vehicle might be a better carrier (Mills & Cross, 2006) than the previously published methimazole PLO gel formulation (Hoffman et al., 2002;

Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006). Our study chose a lipophilic vehicle for the transdermal delivery of methimazole. In addition to the formulation, other variables can affect the transdermal delivery of drugs which include: blood flow to the skin, the skin integrity and hydration. Therefore, the site of application might change the rate of drug delivery, a factor that has been investigated in dogs, but not cats (Mills et al., 2004; Mills et al., 2005; Mills et al., 2006). However, previous studies of transdermal medications in cats have used the inner pinna (Hoffman et al., 2002; Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006) and this same site was selected for this study for that reason.

This 12 week study showed that once daily treatment with transdermal methimazole in a lipophilic vehicle was effective in reducing TT4 concentrations into the reference range which led to improvement in clinical signs (weight gain and reduced blood pressure) in all of the treated cats. At the conclusion of the 12 week study, 14 of the 20 owners of the cats in the transdermal group elected to continue treating their cats with transdermal methimazole. These cats continued to have good control of their hyperthyroidism for up to 2 years, as demonstrated by TT4 concentrations within the reference range and absence of clinical signs in the majority of the rechecks. Previous studies have shown that transdermal methimazole in PLO gel applied twice daily has short term (4–8 weeks) efficacy in treating feline hyperthyroidism (Hoffmann et al., 2003; Lecuyer et al., 2006). However, one study found this treatment less efficacious than oral treatment with methimazole, but resulted in fewer gastrointestinal side effects (Sartor et al., 2004). In contrast, we found once daily dosing with transdermal methimazole in a lipophilic vehicle to be as effective as oral carbimazole in reducing TT4 concentrations.

The starting dose of 10 mg once daily for transdermal methimazole and 5 mg twice daily for oral carbimazole were chosen for this study based on dosage information from

published studies (Mooney et al., 1992; Lecuyer et al., 2006). The two starting daily doses were not equivalent. Carbimazole is a pro-drug of methimazole, with a molecular weight of 186 compared to a molecular weight of 114 for methimazole. Methimazole and carbimazole should be considered equivalent on a molar basis rather than a weight basis, therefore in our study 10mg daily of carbimazole is equivalent to 6 mg of methimazole daily (Jansson et al., 1983). In the current study, the cats in the transdermal group received a higher mean dose of methimazole than the cats in the oral carbimazole group, and this difference may have contributed to the efficacy of the transdermal methimazole.

The serum methimazole concentrations measured in this study correlated poorly with serum TT4 concentrations at the same time point in both treatment groups (Figure 2). Despite wide variation in the serum methimazole concentrations achieved in the study, clinical signs attributable to hyperthyroidism resolved in all treated cats. Previously, it has been shown that only low concentrations of methimazole in the serum were reached when normal cats were treated with methimazole after application to the skin (Hoffman et al., 2002). Pharmacokinetic studies in hyperthyroid and normal cats have shown methimazole to have a short half-life in the plasma (2.3 ± 0.4 h for hyperthyroid cats and 4.7 ± 1.4 h for normal cats) (Trepanier et al., 1991a; Trepanier et al., 1991b). Moreover, a recent study has shown that there is no relationship between the timing of blood sampling after oral methimazole and the TT4 concentration (Rutland et al., 2009). Therefore, since absorbed methimazole concentrates in the thyroid, serum methimazole concentrations are unlikely to be correlated with serum TT4 concentrations. The endpoint of successful treatment for these types of anti-thyroid drugs can be determined by their biological effect, i.e. the decrease in TT4 concentration and resolution of clinical signs rather than the serumconcentration of methimazole alone.

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In this study we collected samples 6–8 h after carbimazole dosing and 18–20 h after transdermal methimazole application. These times were chosen based on the availability of the cats and owners, as the trough dose i.e. a time just prior to the next dose could not be achieved for practical reasons. We found that TT4 concentrations remained suppressed for 18–20 h after (Figure 1) treatment with transdermal methimazole, indicating that for this treatment once-daily application is sufficient. These results are similar to a recent study of healthy cats treated with once daily oral methimazole that showed significant suppression of thyroid hormone concentrations for 24 h (Rutland et al., 2009).

Here we demonstrate that transdermal methimazole delivered in a lipophilic vehicle is safe to administer to cats. Previous studies of cats treated with methimazole have reported side effects of vomiting and diarrhoea (15%) neutropenia (1.5%) pruritus or excoriation of the head (2.3%) and kidney disease (15 to 30%) (Peterson et al., 1988; Becker et al., 2000; Langston & Reine, 2006; Williams et al., 2010). In the present study, one cat in the transdermal group developed acute vomiting which resolved with a change of diet and second cat in the oral group also vomited which resolved with a reduction in the dose of carbimazole. A single cat in the topical methimazole group developed neutropenia which resolved when treatment was stopped for seven days. Six cats (15%) developed clinical signs of kidney disease during the trial, three in the transdermal group and three in the oral group. The rate of side effects observed during this trial was within the range reported in previous studies (Peterson et al., 1988; Becker et al., 2000; Langston & Reine, 2006; Williams et al., 2010) of feline methimazole treatment suggesting that transdermal application of this lipophilic methimazole formulation is a safe treatment for cats.

Owner compliance and satisfaction was higher in the transdermal group than the oral carbimazole group. Identifying barriers to owner compliance is a way of improving adherence to recommendations for medical therapy. Time constraints and convenience are two barriers that have been identified (Abood, 2007). Logically, a medication that is applied once daily is more convenient to owners than twice daily medications. Transdermal medications are also more convenient to owners that have difficulty in administering pills to their cats. When treating hyperthyroid cats consistent adherence and compliance with treatment dosage is important, as the disease is only controlled when the drug is administered at the recommended interval. There are however, disadvantages of transdermal drug delivery. Cutaneous irritation can occur (Riviere & Papich, 2001) and potentially there is increased or inadvertent drug exposure to clients or other animals, which does necessitate the use of non-permeable rubber gloves when administering the drug (Boothe, 2006).

In conclusion, treatment of feline hyperthyroidism with once daily transdermal methimazole in a novel lipophilic vehicle, improved clinical signs, suppressed TT4 and was well tolerated by cats. In addition, owner compliance was higher with once-daily medication applied to the skin compared to twice-daily medication administered per os.

ADDENDUM

After the publication of this paper, in 2011 the United States of America, and in 2013 in New Zealand, Hill's Prescription Diet y/d Feline Thyroid Health was released as an alternative therapy to manage hyperthyroidism. The diet is iodine restricted, and the therapeutic effect is based on the fact that iodine plays an essential role in the synthesis of T3 and T4 (**Chapter 1**, Figure 1.1.3). This iodine restricted diet has been shown to be an effective method in decreasing serum TT4 concentrations in cats with hyperthyroidism (Melendez L.M. et al., 2011a; Melendez et al., 2011b) (van der Kooij et al., 2014). When the low iodine diet is fed exclusively to hyperthyroid cats, by around 8 weeks, between 40 – 75% of cats will have a TT4 concentration within the reference range (van der Kooij et al., 2014; Hui et al., 2015).

Previously, owners who are unable to medicate their cat, and were unwilling or unable to pursue ^{131}I or thyroidectomy, had only one option, transdermal methimazole/carbimazole. With the release of Hill's Prescription Diet y/d Feline Thyroid Health, there are now two options. The major advantage of the low iodine diet is the ease of administration. However, dietary management is not a good option for outdoor cats with access to other iodine sources, for cats that find the food unpalatable or for cats that have other concurrent diseases that require specific diets (such as inflammatory bowel disease). Another issue with the low iodine diet is that poor owner compliance or palatability issues have been reported in around 25% of cats (van der Kooij et al., 2014). The combination of the low iodine diet and anti-thyroid drugs is also not recommended. When selecting a treatment option for cats with hyperthyroidism all treatment options need to be discussed and the pros and cons for each evaluated for the cat and its owner. Which treatment is selected depends on a number of factors including: concurrent disease (chronic kidney disease, diabetes mellitus), age of the cat,

cost, surgical skill, availability of nuclear medicine facilities and the owner's informed opinion. Nutritional management or medical management with oral or transdermal methimazole/carbimazole is often selected when definitive therapy with ^{131}I or thyroidectomy is not possible, due to concurrent nonthyroidal disease, or owner financial constraints.

FOOTNOTES

[a] Norvasc, Pfizer New Zealand Ltd, Auckland NZ

[b] Neo-Mercazole, AFT Pharmaceuticals, Auckland NZ

[c] Advia 120 Automated hematalogy analyser, Bayer, Tarrytown, NY

[d] Hitachi 911, Roche Diagnostics, Tokyo, Japan

[e] Clinitex 50, Bayer using Siemens multistix 10

[f] Immulite, Siemens, Los Angeles, CA, USA

[g] Clavulox palatable drops, Pfizer New Zealand Ltd, Auckland NZ

[h] Hill's ZD, Hill's Pet Nutrition, Inc. Topeka, KS USA

ACKNOWLEDGEMENTS

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Appendix 1: Letter to clinicians explaining the study "The efficacy and safety of transdermal methimazole in the treatment of cats with hyperthyroidism".

Cat were recruited for the study from the Massey University Veterinary Teaching hospital and from local veterinary clinics. Awareness of the study was performed through visiting the local clinics at the start of the study, writing a letter to all local clinicians explaining the study, providing all local clinics with posters to remind them of the study and by regularly faxing through posters with an update on how many cats were still required for the study.

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"The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism."

Kate Hill, Mike Gieseg, Dawn Kingsbury and Paul Chambers

Dear Clinician,

We are starting to recruit hyperthyroid cats for a clinical trial involving a novel transdermal formulation of methimazole. Our pilot study in normal cats has shown that this novel formulation is safe and very effective. The study is outlined below.

Aim: To determine whether once daily administration of a novel transdermal formulation of methimazole is as safe and effective as oral carbimazole in treating naturally occurring cases of hyperthyroidism.

Methods
Forty cats with newly diagnosed, naturally occurring hyperthyroidism will be randomly assigned to receive either oral carbimazole (5mg q12hr) or the novel formulation of transdermal methimazole once daily. Cats will be evaluated at 0, 1, 4, 8 and 12 weeks for a physical examination, body weight, CBC, serum biochemistry, urinalysis, TT4 concentrations and carbimazole/methimazole concentrations. The overall efficacy and any adverse affects of transdermal methimazole will then be compared to the standard treatment.

Inclusion criteria
Newly diagnosed, untreated, naturally occurring hyperthyroidism
Suitable for medical treatment
No significant other medical disease not attributable to hyperthyroidism
Owner willing for rechecks at 0,1,4,8,12 weeks
Visits are performed Monday afternoon between 1-3pm

Exclusion criteria
Previous treatment for hyperthyroidism
Other medical diseases
Owners failing to present for rechecks will be accountable for previous bills
Owner unwilling to pick up medication from MUVTH

Financial assistance
Medication will be provided
Blood work associated with the study will be provided
Consultation fees are covered by the study

For any questions in regards to this study, please contact Dr Kate Hill at 06 356 9099 ext 7448 or email k.hill@massey.ac.nz. Thank you for your help. All cats enlisted will be referred back to their primary veterinarian at the end of the trial.

Kate Hill BVSc (Hons) Dip ACVIM
Registered Specialist in Small Animal Medicine
Senior Lecturer in Small Animal Medicine

Te Kunenga
ki Purehuroa

Appendix 2: Poster provided to all local veterinary clinics to recruit cats for the study "The efficacy and safety of transdermal methimazole in the treatment of cats with hyperthyroidism".

Appendix 3: Example of a facsimile sent to local clinics to help recruit cases for the trial: **"**The efficacy and safety of transdermal methimazole in the treatment of cats with hyperthyroidism".

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FACSIMILE

TO:	[Click **here** and type name]
FAX:	[Click here and type fax number]
FROM:	Dr Kate Hill
DATE:	26 February 2015
SUBJECT:	Hyperthyroid treatment trial
PAGES:	[Click here and type number of pages]

☐ Urgent ☐ For Review ☐ Please Comment ☐ Please Reply

THE RACE IS ON!! PRIZES FOR THE LAST 3 PEOPLE TO SEND ME THE LAST 3 CATS!!!!

A big thank you to everyone who has sent cats in to our study "The efficacy and safety of a novel transdermal forumulation of methimazole in the treatment of cats with hyperthyroidism." already.

I still need <u>**3 more cats**</u> to complete the initial 40 cat trial, so keep them rolling in!

Please keep the poster up and keep reminding your staff. Any questions please call or email me.

Thanks for your help and keep the cats coming!!

Thanks
Kate

Thanks for the referral and please feel free to call with any questions or concerns.

Kate Hill BVSc DipACVIM
Specialist in Canine and Feline Medicine

Appendix 4: **Owner Consent form**, checklist, questionnaire and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Client sticker

Massey University
Veterinary Teaching Hospital

Owner Consent Form

I agree to enter my cat into the study entitled "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism." I have had my cat's condition and the risks and benefits for treatment options explained to me. I understand that participation in this study requires collection of blood samples and urine. I understand that my cat will need to be rechecked in 1 week and then every month for 3 months and failure to do so will result in me being entirely accountable for the first bill.

_____

Owners name

_____   _____

Signature   date

_____

Witness Name

_____   _____

Witness signature   date

Appendix 4: Owner Consent form, **checklist**, questionnaire and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Massey University
Veterinary Teaching Hospital

"The efficacy and safety of transdermal carbimazole in the treatment of cats with hyperthyroidism."

Check list
Week 0

Consent form signed	☐
Client Questions sheet	☐
Physical exam	☐
Body weight	☐
CBC in 0.5ml tube	☐
Serum chemistry 4mls	☐
Total T4	☐
Urinalysis	☐
Urine protein:creatinine ratio	☐
Urine Culture	☐
Blood Pressure	☐

Client sticker

Week 1

Client Questions sheet	☐
Physical exam	☐
Body weight	☐
CBC in 0.5ml tube	☐
Serum chemistry 4mls	☐
Total T4	☐
Urinalysis	☐
Urine protein:creatinine ratio	☐
Blood Pressure	☐

Appendix 4: Owner Consent form, checklist, **questionnaire** and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

<u>**Week 4**</u>

Client Questions sheet ☐

Physical exam ☐

Body weight ☐

CBC in 0.5ml tube ☐

Serum chemistry 4mls ☐

Total T4 ☐

Urinalysis ☐

Urine protein:creatinine ratio ☐

Blood Pressure ☐

<u>**Week 8**</u>

Client Questions sheet ☐

Physical exam ☐

Body weight ☐

CBC in 0.5ml tube ☐

Serum chemistry 4mls ☐

Total T4 ☐

Urinalysis ☐

Urine protein:creatinine ratio ☐

Blood Pressure ☐

<u>**Week 12**</u>

Client Questions sheet ☐

Physical exam ☐

Body weight ☐

CBC in 0.5ml tube ☐

Serum chemistry 4mls ☐

Total T4 ☐

Urinalysis ☐

Urine protein:creatinine ratio ☐

Blood Pressure ☐

Client sticker

Appendix 4: Owner Consent form, checklist, **questionnaire** and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Client Questions

Client sticker

Massey University Veterinary Teaching Hospital

"The efficacy and safety of transdermal carbimazole in the treatment of cats with hyperthyroidism."

Week 0
Since the last recheck, have you noticed your cat doing any of the following

Vomiting   Yes/No
If Yes how often   multiple times a day/daily/few times a week/weekly

Diarrhea   Yes/No
If Yes how often   multiple times a day/daily/few times a week/weekly

Scratching at the ear
Yes/No
If Yes how often   multiple times a day/daily/few times a week/weekly

Not eating
Yes/No
If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has increased
Yes/No
If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has been stable
Yes/No
Lost any weight
Yes/No

Any coat changes
Yes/No

Please describe any other abnormalities

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Appendix 4: Owner Consent form, checklist, **questionnaire** and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

<u>**Nutrition**</u>

Does your cat eat dry food? Yes/No
What brand/s?

_____ -

Does your cat eat canned food? Yes/No
What brand/s?

_____
What percentage of dry:canned food does your cat eat?
100:0 or 50:50 or 25:75 or other_____

Please fill in what your cat ate during these stages of life
<u>**Aged 1-5years**</u>
Brand or Brands _____

_____
Percentage of dry : canned food 100:0 or 50:50 or 25:75 or other_____

<u>**Aged 6-8years**</u>
As above (please circle) or complete the following
Brand or Brands _____

_____
Percentage of dry : canned food 100:0 or 50:50 or 25:75 or other_____

<u>**Aged 9-11**</u>
As above (please circle) or complete the following
Brand or Brands _____

_____
Percentage of dry : canned food 100:0 or 50:50 or 25:75 or other_____

<u>**Aged 11-13**</u>
As above (please circle) or complete the following
Brand or Brands _____

_____
Percentage of dry : canned food 100:0 or 50:50 or 25:75 or other_____

<u>**Aged 14+**</u>
As above (please circle) or complete the following
Brand or Brands _____

_____
Percentage of dry : canned food 100:0 or 50:50 or 25:75 or other_____

Thank you for helping us.
Kate Hill BVSc DipACVIM
Registered Specialist in Small Animal Medicine

Appendix 4: Owner Consent form, checklist, **questionnaire** and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Client Questions

Client sticker

Massey University Veterinary Teaching Hospital
"The efficacy and safety of transdermal carbimazole in the treatment of cats with hyperthyroidism."

Week 1
Since the last recheck, have you noticed your cat doing any of the following

Vomiting   Yes/No
If Yes how often multiple times a day/daily/few times a week/weekly

Diarrhea   Yes/No
If Yes how often multiple times a day/daily/few times a week/weekly

Scratching at the ear
     Yes/No
If Yes how often multiple times a day/daily/few times a week/weekly

Not eating
     Yes/No
If Yes how often multiple times a day/daily/few times a week/weekly

Eating has increased
     Yes/No
If Yes how often multiple times a day/daily/few times a week/weekly

Eating has been stable
     Yes/No

Lost any weight
     Yes/No

Any coat changes
     Yes/No

Please describe any other abnormalities

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Appendix 4: Owner Consent form, checklist, **questionnaire** and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Client Questions

	Client sticker

Massey University Veterinary Teaching Hospital

"The efficacy and safety of transdermal carbimazole in the treatment of cats with hyperthyroidism."

Week 4

Since the last recheck, have you noticed your cat doing any of the following

Vomiting   Yes/No
               If Yes how often   multiple times a day/daily/few times a week/weekly

Diarrhea   Yes/No
               If Yes how often   multiple times a day/daily/few times a week/weekly

Scratching at the ear
               Yes/No
               If Yes how often   multiple times a day/daily/few times a week/weekly

Not eating
               Yes/No
               If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has increased
               Yes/No
               If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has been stable
               Yes/No

Lost any weight
               Yes/No

Any coat changes
               Yes/No

Please describe any other abnormalities

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Appendix 4: Owner Consent form, checklist, **questionnaire** and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Client Questions

Client sticker

Massey University Veterinary Teaching Hospital
"The efficacy and safety of transdermal carbimazole in the treatment of cats with hyperthyroidism."

Week 8
Since the last recheck, have you noticed your cat doing any of the following

Vomiting   Yes/No
                  If Yes how often   multiple times a day/daily/few times a week/weekly

Diarrhea   Yes/No
                  If Yes how often   multiple times a day/daily/few times a week/weekly

Scratching at the ear
                  Yes/No
                  If Yes how often   multiple times a day/daily/few times a week/weekly

Not eating
                  Yes/No
                  If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has increased
                  Yes/No
                  If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has been stable
                  Yes/No

Lost any weight
                  Yes/No

Any coat changes
                  Yes/No

Please describe any other abnormalities

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Appendix 4: Owner Consent form, checklist, **questionnaire** and application instructions for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Client Questions

Massey University Veterinary Teaching Hospital
"The efficacy and safety of transdermal carbimazole in the treatment of cats with hyperthyroidism."

Week 12
Since the last recheck, have you noticed your cat doing any of the following

Vomiting   Yes/No
        If Yes how often   multiple times a day/daily/few times a week/weekly

Diarrhea   Yes/No
        If Yes how often   multiple times a day/daily/few times a week/weekly

Scratching at the ear
        Yes/No
        If Yes how often   multiple times a day/daily/few times a week/weekly

Not eating
        Yes/No
        If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has increased
        Yes/No
        If Yes how often   multiple times a day/daily/few times a week/weekly

Eating has been stable
        Yes/No

Lost any weight
        Yes/No

Any coat changes
        Yes/No

Please describe any other abnormalities

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Appendix 4: Owner Consent form, checklist, questionnaire and **application instructions** for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

Massey University
Veterinary Teaching Hospital

Transdermal Methimazole Application

1. Wipe your cats ear with a dry cotton ball to remove any scale or previous liquid
2. Wear gloves
3. Apply 0.1ml of liquid to your gloved index finger

4. Gently rub onto the inner ear once a day
5. Ears can be alternated daily if you prefer

0.1ml is from 1.0 to 0.9 etc

Appendix 4: Owner Consent form, checklist, questionnaire and **application instructions** for the study "The efficacy and safety of a novel transdermal formulation of methimazole in the treatment of cats with hyperthyroidism".

One cat in the twice daily trial developed slight ear scaling. None of the 18 clinical cats with once daily applications have had any ear problems.

Cat's ear showing scaling after twice daily application of transdermal carbimazole for a week.

Appendix 5: Pathology reports of 3 of the 5 cats who died during the study on the efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism

Cat 1 treated with oral carbimazole for 8 weeks

Species:	Feline	Sex: Female - Desexed	Age: Adult	Breed: DSH

ID:	Foxie	Owner:	Helen
Swainson	Prev. Accn.:	Type:	Post Mortem

HISTORY

The provided history states that this cat was 8 weeks into receiving oral carbimazole (5mg bid) as part of a hyperthyroid treatment trial. It developed intermittent anorexia over the last 4 weeks with weight loss.

GROSS FINDINGS

This cat weighed 2.749kg and while there were large amounts of subcutaneous fat, bony protuberances were prominent. There was bilateral thyroid enlargement; left: 22x12x7mm and right 17x8x5mm. There were 4 small irregular, 2-4mm diameter foci scattered over the visceral pleura. The stomach contained only a small volume of yellow, fluid contents and the colon was distended with similar-appearing contents with slightly thicker consistency. No gross abnormal

HISTOPATHOLOGY

The main histological findings are present and similar in both thyroid glands, in which 60-70% of the parenchyma is replaced by neoplastic branching tubular and follicular structures on a fine fibrovascular stroma. These structures are lined by cuboidal epithelium with pale eosinophilic cytoplasm and rounded nuclei with clumped chromatin. Only small foci of normal follicular structures are present at the periphery of the neoplasm. In the kidneys there are small multifocal areas of interstitial fibrosis with a mild infiltrate of lymphocytes and plasma cells.

DIAGNOSIS

Thyroid glands: bilateral thyroid adenomas
Kidneys; interstitial nephritis, mild, multifocal chronic and lymphoplasmacytic

COMMENTS

Thyroid adenomas in cats are usually functional and result in hyperthyroidism. This probably resulted in the weight loss observed clinically. No hypertrophic cardiomyopathy or aortoiliac thromboembolism was detected.

DISCUSSION WITH PATHOLOGIST

The exact cause of the anorexia and the reason for euthanasia was undetermined from this necropsy examination. A verbal discussion with the pathologist revealed that the cat had moderate dental tartar and kidney disease, however there was no oral ulceration or ulceration in the stomach on pathology examination. The kidney and dental disease was not considered bad enough to cause anorexia. However there was no evidence to suggest that carbimazole treatment was the cause of the anorexia, the exact cause of the anorexia could not be determined from the necropsy examination.

Cat 2 treated with transdermal methimazole

Species: Feline (1)   Sex: Female - Desexed   Age: 11 Years

HISTORY

This cat had reportedly been diagnosed as being hyperthyroid with a heart murmur in June 2007 and was in a transdermal methimazole trial. It was withdrawn from the trial 2-3 weeks ago due to leg weakness.

GROSS FINDINGS

This cat was presented dead. She was judged to be in a poor state of nutrition (body condition score 1/5), adequately hydrated, and in a fair state of preservation. The intestine appeared to be diffusely thickened and contained almost no digesta. The heart had an extremely thickened left ventricular wall with a thin right ventricular wall. The left atrium was dilated. There was a 2 x 2 mm foci of yellowish discoloration in the wall of the left atrium just above the A-V valve. This was interpreted as a resolving thrombus. There was unilateral hyperplasia of the right thyroid gland (1.75 x 1 cm). The left thyroid appeared normal. The kidneys were of normal size. However, on cut surface the cortex was thinner than normal and the medulla had a nodular appearance. There was a small (3 x 2 mm) thrombus in the caudal aorta (at the iliac bification) which could have explained hind limb weakness.

HISTOPATHOLOGY

Replacing approximately 70% of normal gland architecture is a moderate to densely cellular mass with little fibrous connective tissue. The cells of the mass are composed of cuboidal to columnar cells with indistinct borders with moderate amounts ofeosinophilic cytoplasm and darkly staining round to oval nuclei. There is less than one mitotic figure per high powered field. Approximately 20% of the gland is cystic with abundant brightly eosinophilic secretory material containing cholesterol crystal spaces. All the bronchioles show hyperplasia of the epithelium. Sections of pleura show a massive accumulation of macrophages, some multinucleated, with a large amount of fine to foamy vacuolation and an eosinophilic cytoplasm Macrophage nuclei are variable in size, shape and staining from irregular to rounded, from dark to lightly stained. The kidney contained a focal linear interstitial cellular infiltrate, reaching from the capsule to about one third of the way into the cortex.

DIAGNOSIS

Heart. Hypertrophic cardiomyopathy (gross observation).

Thyroid gland. Benign adenomatous hyperplasia with cystic dilation.

COMMENTS

No reason for the death of this cat could be identified on necropsy examination. However, it is possible that an undetected thromboembolic episode due to the cardiomyopathy could have occurred. The hypertrophic cardiomyopathy in this case was most likely secondary to hyperthyroidism. The changes in the lung are likely to be incidental and age related.

DISCUSSION WITH PATHOLOGIST

A verbal discussion with the pathologist discussed that the cat was euthanized, which was the cause of death. The necropsy was performed 3 days after euthanasia. The embolism in the caudal aorta may have decreased in size over that time, and originally extended further into the iliac bification. The cardiac changes combined with the evidence for a resolving thrombus and the small thrombus in the caudal aorta, suggest that cardiac disease secondary to hyperthyroidism were the likely cause of the weakness, and there was no evidence to suggest methimazole was the cause of the signs.

Cat 3 treated with transdermal methimazole

Species: Feline (1) Sex: Female - Desexed Age: 13 Years Breed: Oriental Shorthair ID: Cleopatra

HISTORY

Cleopatra had a history of hyperthyroidism and renal disease. She was part of a transdermal methimazole research trial for the treatment of hyperthyroidism, but 6 weeks into the trial developed tachypnoea and a pleural effusion. 400mL of mildly chylous pleural fluid was drained (80% small lymphocytes and small numbers of large, probably lymphoblastic, cells). No cardiac murmur was detected. She was euthanased.

GROSS FINDINGS

The cat was presented dead, in very thin body condition (body condition score 2/9, bodyweight 2.7kg) and a very good state of preservation. Significant findings were present in the thoracic cavity and the thyroid glands.

 The cranial thoracic mediastinum contained a large (approximately 8 x 4 x 3cm), multinodular, moderately firm, cream-yellow mass that surrounded and compressed blood vessels and the distal trachea and displaced the lungs caudally. Several small (<1cm diameter) round nodules similar in appearance to the thoracic mass were present within the peritracheal connective tissue. Neither thymic structures nor mediastinal lymph nodes could be located. The thyroid glands were both moderately enlarged (left 2.5cm x 0.8cm; right 1.5cm x 0.7cm) and multinodular and the parathyroid glands were mildly enlarged. The liver had a mildly increased lobular pattern but its size and shape were unremarkable. The heart was grossly unremarkable.

HISTOPATHOLOGY

Sections from multiple organs were examined.Cranial thoracic mass and peritracheal nodules: This mass consists of a partially encapsulated, well-demarcated, densely cellular, expansile neoplasm composed of sheets of round cells in a moderate fibrovascular stroma and divided into irregular, poorly defined lobules by fibrous trabeculae. Neoplastic cells are markedly pleomorphic but are often large with distinct borders, scant to small amounts of pale eosinophilic cytoplasm and large round basophilic nuclei with coarsely granular chromatin and a single prominent magenta nucleolus. The mitotic rate is high, averaging 35-40 mitoses per 10 high power fields.

Moderate numbers of scattered mature lymphocytes and multifocal areas of necrosis are present within the mass. The small nodules within the peritracheal connective tissue contained similar neoplastic cells to those present in the main thoracic mass.

On immunohistochemistry, neoplastic cells demonstrated cytoplasmic reactivity with antibodies against vimentin and CD3 but did not with antibodies against CD79a and AE1/AE3 (suggestive of T-cell lymphosarcoma).

Thyroid glands: The thyroid glands contain multifocal, well-demarcated, unencapsulated, densely cellular oval masses composed of uniform cuboidal to polygonal cells arranged in nests and follicles supported by a fine fibrovascular stroma. Moderate amounts of brightly eosinophilic secretory material (colloid) are present within some follicular remnants. Cells have distinct borders, moderate amounts of pale eosinophilic granular cytoplasm, and round, often basilar, nuclei with finely stippled chromatin and often a single nucleolus. Both glands contain several small cysts which are lined by flattened cuboidal epithelial cells and contain small amounts of eosinophilic material (protein).

Parathyroid glands: There is mild hyperplasia of both glands.

Kidneys: The cortical interstitium contains multifocal small to medium sized well-demarcated infiltrates of lymphocytes and plasma cells with moderate fibrosis, often surrounding atrophic tubules. Diffusely, moderate numbers of tubules have vacuolated, attenuated or occasionally degenerating epithelium and contain intraluminal eosinophilic fluid (protein) and occasional debris (casts). Glomeruli are occasionally sclerotic. The medulla shows mild diffuse interstitial fibrosis. Moderate numbers of lymphocytes are present within and under the renal pelvic epithelium.

Liver: In many areas, centrilobular sinusoids are mildly congested and moderate numbers of centrilobular hepatocytes and Kupffer cells contain orange-brown granular pigment (haemosiderin or bile). Many periportal areas contain low numbers of lymphocytes and plasma cells and occasional neutrophils. A single small focal area within the parenchyma contains necrotic hepatocytes and moderate numbers of neutrophils and macrophages. Many randomly scattered microgranulomas are present. Sections of spleen, heart and lung do not contain significant findings.

CLINICAL PATHOLOGY

Urinalysis revealed dilute urine (USG =1.014), very mild proteinuria and biliribinuria and marked haematuria.Fine needle aspirates and impression smears from the cranial

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thoracic mass contained large numbers of ruptured cells and occasional intact round cells with large round basophilic nuclei and small amounts of pale eosinophilic cytoplasm occasionally containing small brightly eosinophilic granules.

DIAGNOSIS

Cranial thoracic mass: Poorly differentiated T-cell lymphosarcoma (see comments below).

Thyroid gland: Hyperplasia, bilateral, nodular, moderate, chronic (benign adenomatous hyperplasia).

Parathyroid glands: Hyperplasia, bilateral, diffuse, mild, chronic (renal secondary hyperparathyroidism).

Kidneys: Interstitial and pyelonephritis, bilateral, multifocal, moderate, chronic and lymphoplasmacytic with interstitial fibrosis, tubular atrophy and intratubular protein (chronic renal disease).

Liver: Centrilobular congestion, mild with bile or haemosiderin retention and mild lymphoplasmacytic pericholangitis.

Abdominal fat and connective tissue: Icterus, diffuse, mild.

COMMENTS

Kate, although the cell morphology is not entirely typical, the location of the tumour within the cranial thorax together with histological and immunohistochemical findings indicate a poorly differentiated T-cell lymphosarcoma.

Histology confirms the presence of bilateral benign adenomatous thyroid hyperplasia and chronic renal disease.

Cleopatra's pleural effusion most likely resulted from a combination of decreased lymphatic and venous return due to the compressive effects of the tumour. At the time of post mortem the tumour did not appear to be significantly impeding blood flow from the heart, as there was no evidence of pulmonary oedema and only mild passive congestion of the liver. Evidence of mild pericholangitis and probable bile retention is present in the liver, which is reasonably common in older cats and may account for the icterus seem at post mortem.
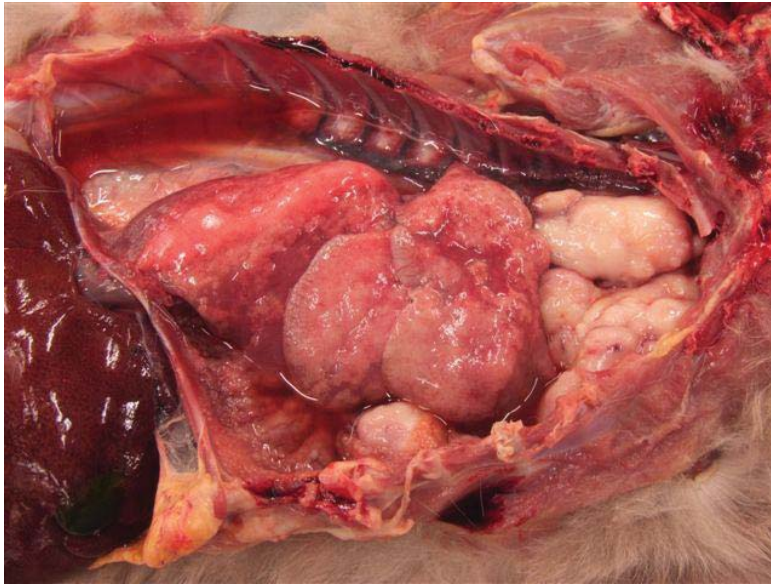
Figure 1: Mediastinal lymphoma in cat 3 who died during the study on the efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism.

Figure 2: Mediastinal lymphoma after it has been removed from the thorax from cat 3 who died during the study on the efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism.

Appendix 6: Complete history and long term follow up of the one cat in the study on the efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism, who developed neutropenia and was treated with transdermal methimazole once a day.

Case History

A 15 year old male neutered domestic short haired cat (body weight 2.9 kg) was presented for inclusion into a trial involving a novel lipophilic transdermal methimazole with weight loss and polyphagia (Day 0). Clinical examination revealed a cat with a body condition score of 3/9, goitre, one eye and cropped ears from previous surgery to remove a squamous cell carcinoma. A CBC, serum chemistry, urinalysis, urine culture and total thyroxine (TT4) concentration (176 nmol/L range 20-40 nmol/L) (Table 1) was performed and hyperthyroidism was diagnosed. The cat was treated with 10 mg of the novel lipophilic transdermal methimazole applied to the pinnae once daily.

The cat was rechecked 7 days later, a CBC, serum chemistry, urinalysis and TT4 were repeated, and the TT4 concentration had decreased to just above the reference range (Table 1).

At day 30 after commencing the methimazole, the cat was rechecked again. The owner reported the cat to be healthy and the cat was eating less and had gained 150 grams (3.05 kg). A CBC revealed severe neutropena (0.27×10^9). The cat was normothermic (T=38.0° C) and the clinical examination was normal. A urine culture revealed no growth of bacteria, and FIV, FeLV tests were negative. The cat was clinically normal and never developed a fever. Transdermal methimazole was stopped for 7 days. Prophylactic amoxicillin-clavulonic acid[c] (20mg/kg PO BID) (Clavulox palatable drops, Pfizer New Zealand Ltd, Auckland NZ) was administered. The cat was monitored with

clinical evaluations and blood work at day 33, 37, and 44 (see Table 1). At day 37, the

cat was demonstrating severe signs of hyperthyroidism (polyuria, polydipsia and

polyphagia) and the TT4 was severely elevated (>193 nmol/L, reference range 20-40

nmol/L). Transdermal methimazole was reinstated at half the original dose (5 mg once a

day), amoxicillin-clavulonic acid was continued. At day 44, the neutrophil count had

returned to normal, antimicrobial therapy was discontinued.

The cat was re-evaluated at 8 & 12 weeks and then every 3 months for a further until

13 months until the cat died of recurrence of the squamous cell carcinoma. No further

reactions to the methimazole were noted. The methimazole concentration at the time of

toxicity was 0.17 µg/mL, subsequent methimazole concentrations ranged from

0.07-0.12 µg/mL (Table 1). Methimazole concentrations did not correlate to the

development of toxicity and did not correlate to the TT4 concentrations. The cat was

monitored for 13 months until euthanasia for the re-occurrence of squamous cell

carcinoma. No further methimazole toxicity developed during this 13 month period.

Table 1: Haematology and serum biochemistry results from a 15 year old male neutered domestic short haired cat that developed neutropenia after therapy with once daily administration of a novel formulation of transdermal methimazole.

	Units	Ref range	week 0	week 1	week 4	day 33	week 5	week 6	week 8	week 12	6 mths	7 mths	9 mths	11 mths	12 mths	13 mths
weight	kg		2.9	3.09	3.05		3.1		3.03	2.93	3.14	3.48	4	4.4	4.5	
HR	beats/min		240	200	160		200		160	160	160	160	190	190	168	
BP	mmHg		200	170	120				130	160	160		140	180	187	
CBC																
RBC	X10^{12}/L	5-10	8.6	7.6	7.6	7.6	7.2	7.2	7.2	8.9	6.7	6.5	8.4	7.6		
Hg	g/L	80-150	133	118	120	123	115	115	116	125	109	106	127	117		
HCT	L/L	0.24-0.45	0.4	0.37	0.37	0.37	0.35	0.35	0.36	0.37	0.34	0.32	0.39	0.35		
MCV	fl	39-55	47	49	49	49	49	49	50	47	50	49	46	47		
MCH	pg	13-18	15	16	16	16	16	16	16	16	16	16	15	15		
MCHC	g/L	290-360	329	319	324	331	325	331	322	338	323	331	329	331		
Platelets	X10^9/L		578	614	656	634	489	405	490		675	594	482			
WBC	X10^9/L	5.5-19.5	10.8	8.8	1	1.2	3	7.3	4.4	9.1	12.4	11.2	7.9	8.3		
Seg Neut	X10^9/L	2.4-12.5	8.21	6.16	**0.27**	**0.16**	**0.54**	3.87	2.73	7.01	10.2	8.06	5.8	6.6		
Lymphs	X10^9/L	1.5-7.0	1.84	1.94	**0.66**	**0.88**	**2.07**	2.12	0.84	1	1.7	2.69	1.9	1.4		
Monocytes	X10^9/L	0-0.9	0.32	0.26	0.07	0.02	0.21	0.51	0.09	0.46	0.2	0.34	0.2	0.2		
Eosinophils	X10^9/L	0-1.5	0.43	0.44		0.14	0.18	0.8	0.75	0.64	0.2	0.11	0.2	0.2		

Table 1 (continued): Haematology and serum biochemistry results from a 15 year old male neutered domestic short haired cat that developed neutropenia after therapy with once daily administration of a novel formulation of transdermal methimazole.

	Units	Ref.														
ALP	IU/L	0-85	81	64	95	68	65	69	69	63	46	29	37	26	24	35
ALT	IU/L	0-100	264	152	501	305	291	554	215	156	117	53	68	50	55	111
TP	g/L	54-78	74	69	69	69	70	64	66	69	72	68	82	82	85	85
Albumin	g/L	21-38	34	34	34	34	33	32	33	33	32	32	37	37	39	35
Globulin	g/L	26-51	40	35	35	35	37	32	30	34	40	36	45	45	46	50
A/G	ratio	0.5-1.1	0.85	0.97	1	0.97	0.9	1	1	1.03	0.8	0.89	0.82	0.8	0.85	0.7
Urea	mmol/L	6-11.8	7.8	8.5	7.2	8.8	8.7	10.1	9.3	8.5	11.7	9.6	8.7	11.4	12.6	7.3
Creatinine	umol/L	80-178	68	65	70	93	61	95	88	68	80	87	126	111	104	93
Calcium	mmol/L	1.81-2.7	2.5	2.27	2.3	2.42	2.32	2.23	2.41	2.29	2.39	2.25	2.36	2.37	2.52	2.51
Sodium	mmol/L	147-156	161	155	152	154	152	152	153	149	152	151	152	149	157	153
Potassium	mmol/L	3.5-5.0	4.5	4.3	5.3	4.3	4.2	4.5	4.4	5.1	4.2	4.5	4.1	4.4	4.6	4.4
Chloride	mmol/L	108-128	117	114	118	116	118	120	118	118	115	114	115	115	119	115
Phosphate	mmol/L	1.3-2.8	1.83	1.54	1.6	1.44	1.48	1.93	1.57	1.62	1.9	1.54	1.46	1.11	1.46	1.63
Glucose	mmol/L	3.9-6.1	4.8	4.8	4.8			4.4	4.7	5	5.5	4.5	9	5.9	5.7	5.4
TT4	nmol/L	20-40	176	42	27	ND	>193	57	49	26	112	13	59	6	10	45
[MMI]	µg/mL		<0.06	0.08	0.17	ND	<0.06	0.07	0.1	0.08	0.12	0.05				
USG			1.024	1.011	1.02		1.027	1.022	1.022	1.018	1.05	1.056		1.051		
Med						60 mg A-clav	60 mg A-clav									
MMI dose			10 mg	10 mg	10 mg	0	5 mg	5 mg	5 mg	5 mg	10 mg	10 mg	15 mg	12 mg	10 mg	10 mg

USG = Urine specific gravity TT4 = serum Total thyroxine concentrations MMI = methimazole Med = medications A-Clav = Amoxicillin – clavulonic acid

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Chapter 5

Percutaneous absorption of methimazole: an *in vitro* study of the absorption pharmacokinetics for two different vehicles

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PREFACE

The study in **Chapter 3** of this thesis revealed that serum concentrations of methimazole were measurable in healthy cats following the application of 5 mg and 10 mg of methimazole in a lipophilic vehicle for seven days to the inner pinna. **Chapter 4** investigated the *in vivo* efficacy and safety of methimazole in a lipophilic vehicle applied to the inner pinna of client owned hyperthyroid cats. However, neither of these studies confirmed that methimazole was actually absorbed through the pinnal skin, as the drug could have been ingested through grooming. Therefore, the study in this chapter characterizes the percutaneous absorption pharmacokinetics of methimazole in a novel lipophilic vehicle compared to the application of methimazole in PLO gel on feline ear tissue using a finite dose in an *in vitro* Franz cell model. Further information on the materials and methods, which may have been removed for the publication of this chapter as a manuscript, can be found in **Appendix A.**

This chapter was accepted to the *Journal of Veterinary Pharmacology and Therapeutics* and is formatted in the style of this journal.

ABSTRACT

The use of transdermal medications in cats has become popular in veterinary medicine due to the ease of administration compared to oral medication. However, the research to support systemic absorption of drugs applied to the pinna after transdermal administration in cats is limited. The aim of the current study was to characterize the percutaneous absorption pharmacokinetics of methimazole in a lipophilic vehicle compared to methimazole in Pluronic® lecithin organogel (PLO) using a finite dose applied to feline ear skin in an *in vitro* Franz cell model. The two formulations of methimazole (10 mg) were applied to the inner stratum corneum of six pairs of feline ears. The receptor medium was sampled up to 30 hours post administration and methimazole concentrations were measured using high performance liquid chromatography (HPLC). Histological examination of all ears was undertaken as small differences in the thickness of ear skin may have contributed to inter-individual differences in methimazole absorption between the six cats. Methimazole was absorbed more completely across the pinnal skin when administered in the lipophilic vehicle compared to administration in the PLO gel ($p<0.001$).

Keywords: transdermal, methimazole, cats, *in vitro*

INTRODUCTION

Methimazole is an inhibitor of thyroid hormone synthesis and is commonly used to treat cats with hyperthyroidism (Peterson et al., 1988; Trepanier, 2007; Frenais et al., 2009; Mooney, 2010). Cats can be difficult to medicate a factor that supports the use of a formulation of methimazole compounded for transdermal application which may have higher owner compliance than administration in a tablet.

To date, there are limited studies available on the use of transdermal methimazole in cats, with two pharmacokinetic studies (Hoffman et al., 2002; Hill et al., 2014) and a handful of clinical trials undertaken in hyperthyroid cats (Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006; Hill et al., 2011; Boretti et al., 2013). The pharmacokinetics of transdermal methimazole in Pluronic® lecithin organogel (PLO) gel has been reported in six normal cats with only two cats having detectable concentrations of methimazole in the serum in the first 24 h after administration (Hoffman et al., 2002). However, when the pharmacokinetics of 10 mg of methimazole administered in a lipophilic vehicle was performed in 5 normal cats, all had detectable serum concentrations of methimazole within the first 24 h (**Chapter 3**) (Hill et al., 2014). Transdermal methimazole has been shown to be an effective treatment for cats with hyperthyroidism (Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006; Hill et al., 2011; Boretti et al., 2013).

It should be noted that serum concentrations of methimazole do not correlate with efficacy (total thyroxine (TT4) concentration changes) since methimazole accumulates in the thyroid gland in cats (**Chapter 4**) (Hill et al., 2011). For example, methimazole has a short half-life in cats (2.3 ± 0.4 h for hyperthyroid cats and 4.7 ± 1.4 h for normal cats), yet TT4 concentrations, the clinical end-point, only start to decrease 24 h following administration. However, irrespective of the drug concentrations in serum

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after transdermal administration there is uncertainty following topical application of methimazole as to whether the drug penetrates solely through the skin (or the ear in this case) or if there is some degree of oral ingestion after self-grooming (Hoffmann et al., 2003). An *in vitro* model would answer this question and also compare the effects of formulation on the rate and extent of the penetration of transdermal methimazole applied to the ears of cats. The aim of the current study was to characterize the percutaneous absorption pharmacokinetics of methimazole in a novel lipophilic vehicle compared to the application of methimazole in PLO gel on feline ears using a finite dose in an *in vitro* Franz cell model.

MATERIALS AND METHODS

Animals

Six domestic short haired cats (two mature adult males, three young adult females and one young adult male cat) at a local pound were euthanased by an intravenous injection of sodium pentobarbital and the pinna harvested soon after death. The ears were frozen at -20°C until required (the two older male cat ears were frozen for five months, and the remainder were frozen for five days). This experimental protocol was approved by the Animal Ethics Committee of the University of Queensland (approval number SVS/494/12).

Ear skin was defrosted and transected with gentle blunt dissection to separate the ventral (inner surface) from the dorsal aspect of the ear. Subcutaneous tissue and cartilage tissue were removed. The inner pinnal skin was rinsed with tap water to remove any blood and then dried gently with a gauze swab. One circular section of skin measuring 2 cm^2 diameter was cut from each inner pinnal section cut using a round biopsy punch. The remainder of the ear skin was trimmed for histology to determine epidermal and dermal thickness.

Histology

Samples of the skin surrounding the hole left from the biopsy punch were trimmed and fixed in 10% buffered formalin for 24–48 h, processed and embedded into paraffin blocks. Haematoxylin and eosin (H&E) staining was performed on 3 µm sections of tissue cut from the formalin-fixed, paraffin-embedded (FFPE) blocks for light microscopy.

Histological analysis was performed using a Nikon Eclipse N*i* microscope. Histological images were captured using NIS Elements software (Nikon Instruments Inc., Melville,

NY, United States) and analysed using Image J software (Schneider et al., 2012). For each sample, the inner stratum corneum, inner epidermis, number of nucleated epidermal cell layers, inner dermis and pinnal cartilage were measured or counted at three randomly selected locations and the measurements averaged as previously described (Monteiro-Riviere et al., 1990). Both the left and right ears of each cat were examined

In vitro skin penetration studies

Ear skin from the inner pinnae were mounted onto Franz-type diffusion cells with the stratum corneum uppermost as previously described (Mills et al., 2005). The surface area of ear skin exposed to drug in the diffusion cells was 1.13 cm^2. The dermal chamber was filled with 3.5ml of a reservoir solution suitable for a lipophilic drug of phosphate-buffered isotonic saline (sodium chloride 8.0 g/L, potassium chloride 0.2 g/L, disodium hydrogen phosphate 1.15 g/L, potassium dihydrogen phosphate 0.2 g/L) (PBS)(MP Biomedicals, Sydney Australia) with 4% bovine serum albumin (BSA) (Sartorelli et al., 2000), pH 7.4±0.1 and the donor compartment left open to ambient laboratory air conditions. All cells were mounted in a diffusion apparatus with the dermal bathing solution being magnetically stirred and the temperature of the skin in the diffusion cell was maintained at approximately 32°C. After mounting, the skin was hydrated for an hour with sodium phosphate buffer (0.1 M) and the system was allowed to equilibrate. The buffer solution was aspirated from the surface of the ear skin and the formulations of methimazole applied. A commercial preparation of lipophilic transdermal methimazole (0.1 mL/10 mg) and prepared methimazole in PLO gel (0.1 mL/10 mg) was applied to the inner pinnal skin, using 1 mL syringes, pre weighed to ensure accurate application of 0.1 mL. Each application was spread across the inner pinnal skin with a glass rod. For each of the six cats, the methimazole in PLO was

applied to the left inner pinnal skin, and the methimazole in the lipophilic vehicle applied to the right inner pinnal skin. The donor compartment was left open to ambient laboratory conditions, to simulate conditions required for the natural absorption of the drug.

Both the lipophilic and PLO gel formulations were applied as finite doses, where a limited amount of the each formulation is applied to the skin surface, simulating conditions for the *in vivo* patient. The OECD Guideline 428 and Guidance Document 28 (OECD, 2004a; OECD, 2004b), define finite dose absorption experiments as the application of ≤ 10 µL/cm^2 of a liquid formulation to the skin. For semi-solid and solid substances, values range between 1 and 10 mg/cm^2. In this study, 10 mg of methimazole was applied to the skin, with a skin surface area of 1.13 cm^2 (8.8 mg/cm^2), criteria which would meet conditions for a finite dose under OECD guidelines. Therefore the cumulative methimazole concentration and absorption curves were analysed for finite dose conditions.

Sampling time and sample collection

A 200 µL sample of reservoir solution was removed at 1, 2, 4, 6, 8, 12, 18, 24 and 30 h and 200 µL of fresh solution was replaced into the sampling port. The 200 µL aliquots were frozen at -20°C until subsequent analysis, within two months of sampling.

In vitro retention

At the end of the experiment, the Franz cells were dismantled; the skin removed, rinsed thoroughly with tap water and patted dry with gauze swabs. The skin exposed to the formulations was excised and was then macerated with scissors. Macerated samples were placed in pre-weighed vials, and re-weighed. Samples were labelled and frozen at -20 °C until required for further analysis.

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Chemicals

Chemicals to make the Methimazole in PLO were purchased from Sigma Aldrich, St Louis MO USA. Methimazole in a lipophilic formulation was supplied by Bayer, NZ. (Batch number NZ05784, Date of manufacture December 2012, Expiry December 2013).

Bovine serum albumin was purchased from Trace Biosciences (Auckland, New Zealand), PBS from MP Biomedicals (Sydney, Australia)

Methimazole formulation

Two formulations of methimazole were tested. Methimazole in Pluronic® lecithin organogel at a concentration of 100 mg/mL (Date of manufacture 20/5/13) and a commercial lipophilic formulation supplied by Bayer NZ Ltd, Auckland New Zealand (Hyper-T™ Earspot: Batch number NZ05784, Date of manufacture Dec 2012, Expiration Dec 2013, date of study 2 June 2013).

Lipophilic formulation of transdermal methimazole

Methimazole for transdermal application was formulated by Bayer NZ Ltd, United States patent number US 2010/0137389 at a concentration of 100 mg/mL (Nanjan et al., 2010) and previously described (**Chapter 4**) (Hill et al., 2011).

Pluronic® lecithin organogel

Lecithin/isopropyl palmitate solution was prepared using 100 g of lecithin (soya granular), 100 g of isopropyl palmitate, and 0.66 g of sorbic acid FCC powder. Pluronic® gel 20% was prepared with 20 g of Pluronic® F127 NF, 0.3 g of potassium sorbate NF, and purified water, q.s. to 100 mL. Transdermal methimazole gel was formulated using 0.3 g of methimazole USP, 0.66 mL of lecithin/isopropyl palmitate solution, and Pluronic® F127 gel 20%, q.s. to 3.0 mL. The resulting gel contained 10 mg/0.1 mL. Gel was prepared for use within one week of application.

Analytical methods

The methods for high performance liquid chromatography (HPLC) analysis of methimazole from serum have been previously described (**Chapter 4**) (Hill et al., 2011).

Samples were analysed for methimazole by a Waters HPLC (600 Model controller with 717 plus auto sampler and a 2998 Model photodiode array detector). For each run, 10 µL was injected at a flow rate of 0.6 mL/min onto the column (Phenomenex Luna C18, 150 x 4.6 mm, 5 µm, with a guard column) at 30°C. The detection wavelength was 252 nm, run time 15 minutes. The data was processed and integrated with Waters software (Empower™ 2) (Waters Corporation; Milford, MA, USA). Methimazole standards were run before all samples were analysed. Limit of detection (LOD) was 20 ng/mL; limit of quantification (LOQ) was 70 ng/mL, the precision was 1%. Frozen samples were thawed to room temperature and 90 µL was taken and mixed with 10 µL protein extraction solution. The protein extraction solution was made of 0.1 g/mL of 5-sulfosalicylic acid in 60% water and 40% acetonitrile.

The samples were vortexed and centrifuged at 14000g for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of 100 µL was analysed.

For the validation of the procedure, aliquots (0.1 mL) of receiver medium (PBS and BSA) were spiked with standard solutions of transdermal methimazole in lipophilic vehicle and PLO gel.

Skin Extraction

Frozen macerated skin samples were thawed to room temperature and 1 mL of water added. The sample was left for 24 hours at 4°C for methimazole desorption.

The sample liquid was taken (90 µL) and mixed with 10 µL protein extraction solution. The protein extraction solution was made of 0.1 g/mL of 5-sulfosalicylic acid in 60% water and 40% acetonitrile.

The samples were vortexed and centrifuged at 14 000 g for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of the supernatant (100 µL) was collected and analysed by the HPLC as described above.

Recovery efficiency validation

The amount of methimazole in the lipophilic vehicle and methimazole in PLO gel extracted from the skin was calculated from blank skin samples spiked with known amounts of methimazole in each vehicle. Ten skin samples where spiked with methimazole in PLO or lipophilic vehicle at concentrations of 0.01 mg, 0.1 mg, 0.5 mg, 1 mg and 5 mg. Samples were left at room temperature (25°C) for 24 hours for absorption of methimazole. The methimazole was then extracted from the skin as described above and a percentage recovery determined which was then used to adjust

concentrations extracted from the skin after application of methimazole in either vehicle formulation. The amount of methimazole recovered from the skin represented the potential absorbable dose of methimazole.

Data and statistical analyses

The concentration of methimazole in the receptor fluid at each time point was measured. To calculate the true methimazole concentration at each time point, a correction factor was applied which accounted for the dilution effect of 200 µL being removed from the 3.5 mL chamber and replaced with 200 µL of fresh PBS and BSA:

(Measured HPLC concentration) Concentration at time 1 = x µg/mL

Diluted concentration at time 1 after 200 µL is replaced $= x \times \frac{3.3}{3.5}$

(Measured HPLC concentration) Concentration at time 2 = y µg/mL

True (corrected for dilution at time1) concentration at time 2 $= y + \left[x - x \times \frac{3.3}{3.5} \right]$

The flux (J) was calculated using the formula

$\qquad$ J = Q/(At) (Franz et al., 2009)

Where Q is the total quantity of compound traversing the membrane in time t, and A is the area of exposed membrane in cm^2. For this experiment the area was constant at A = 1.13 cm^2.

The cumulative amount of methimazole that had permeated the skin into the receptor fluid was calculated for each replicate at each sampling time point. Individual

cumulative methimazole data is provided as well the calculated geometric mean with 95% confidence intervals for cumulative methimazole and flux.

The mean total absorption of methimazole was calculated from the addition of the methimazole concentration in the receptor fluid (absorbed dose of drug) to the methimazole recovered from the skin samples (absorbable dose of drug). Data is shown as geometric mean and 95% confidence intervals.

Linear regression models were created to test the difference between groups for both cumulative amount of methimazole and flux, with the group and time as fixed effects; an interaction between group and time was tested. Models were run in Stata version 12 (Statacorp LP, College Station, Texas). Mean total absorption of methimazole at the completion of the experiment was compared using Student t-test. A value of $p < 0.05$ was considered significant. Statistical analysis of the cumulative methimazole concentrations divided into age groups was not performed due to the small sample size.

RESULTS

The cumulative methimazole concentration over time and flux is shown in Figures 1 and 2. Individual cat data are shown in Figure 3. The methimazole in a lipophilic vehicle had a significantly greater cumulative concentration ($p < 0.001$) and rate of absorption ($p < 0.001$) compared to the methimazole in the PLO gel.
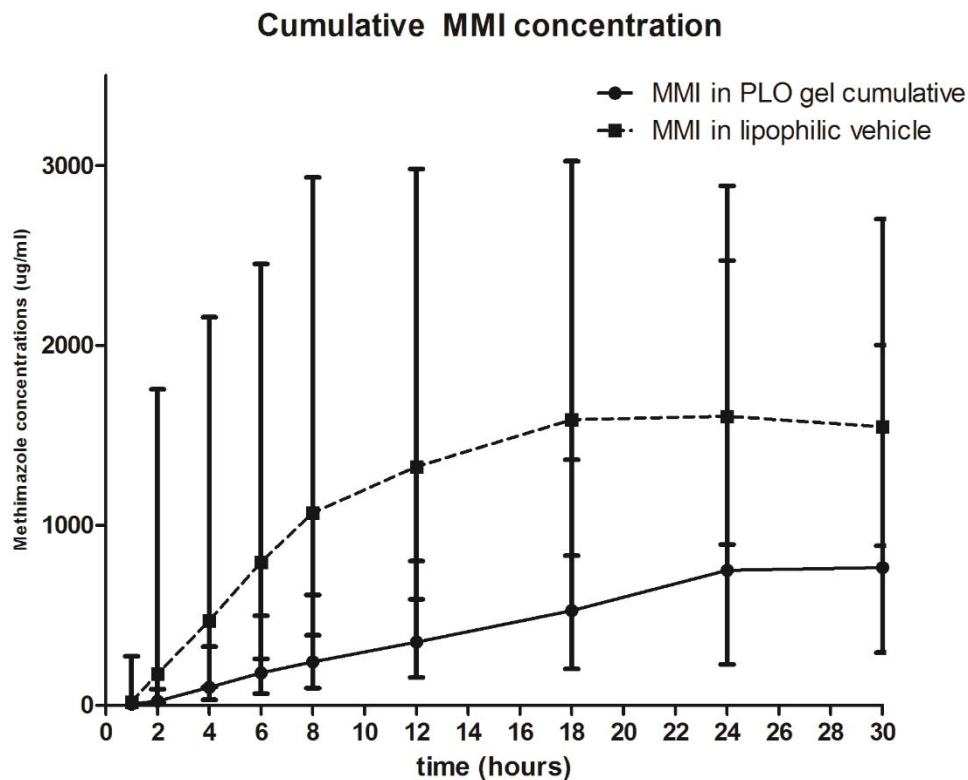
Figure 1: Cumulative methimazole (MMI) concentration after the application of 10 mg of MMI in Pluronic® lecithin gel (PLO) to the left inner pinna skin, or MMI in a lipophilic vehicle to right inner pinna skin of six pairs of inner pinna skin discs using an *in vitro* Franz cell model. The error bars represent 95% confidence intervals. $p < 0.001$
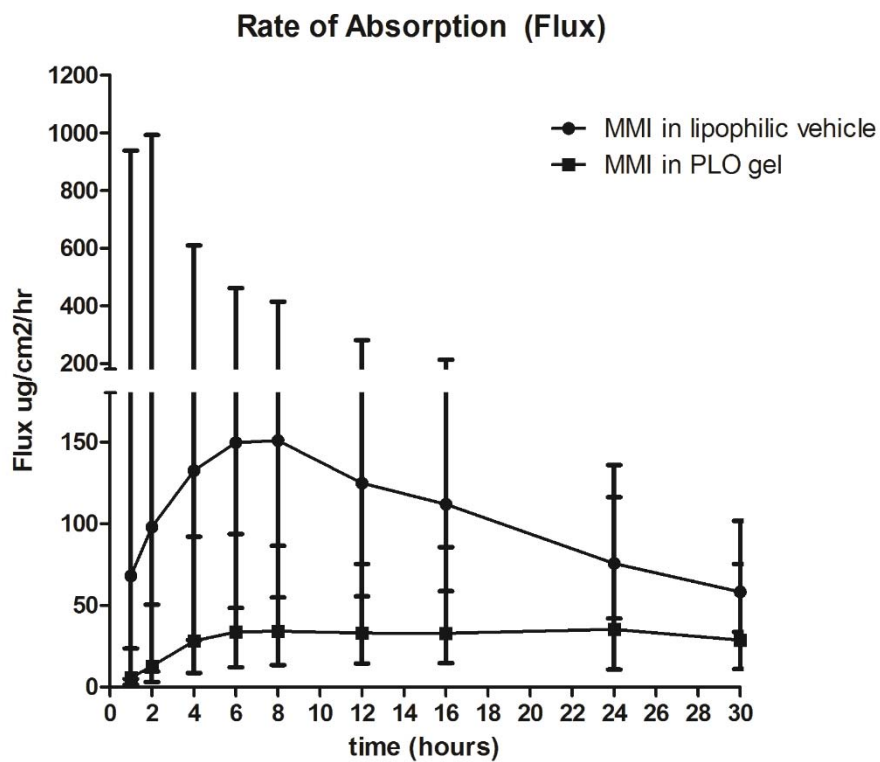
Figure 2: Rate of absorption (Flux) after the application of 10mg of methimazole (MMI) in Pluronic® lecithin gel (PLO) to the left inner pinna skin, or MMI in a lipophilic vehicle to right inner pinna skin of six pairs of inner pinna skin discs using an *in vitro* Franz cell model. The error bars represent 95% confidence intervals. $p < 0.001$
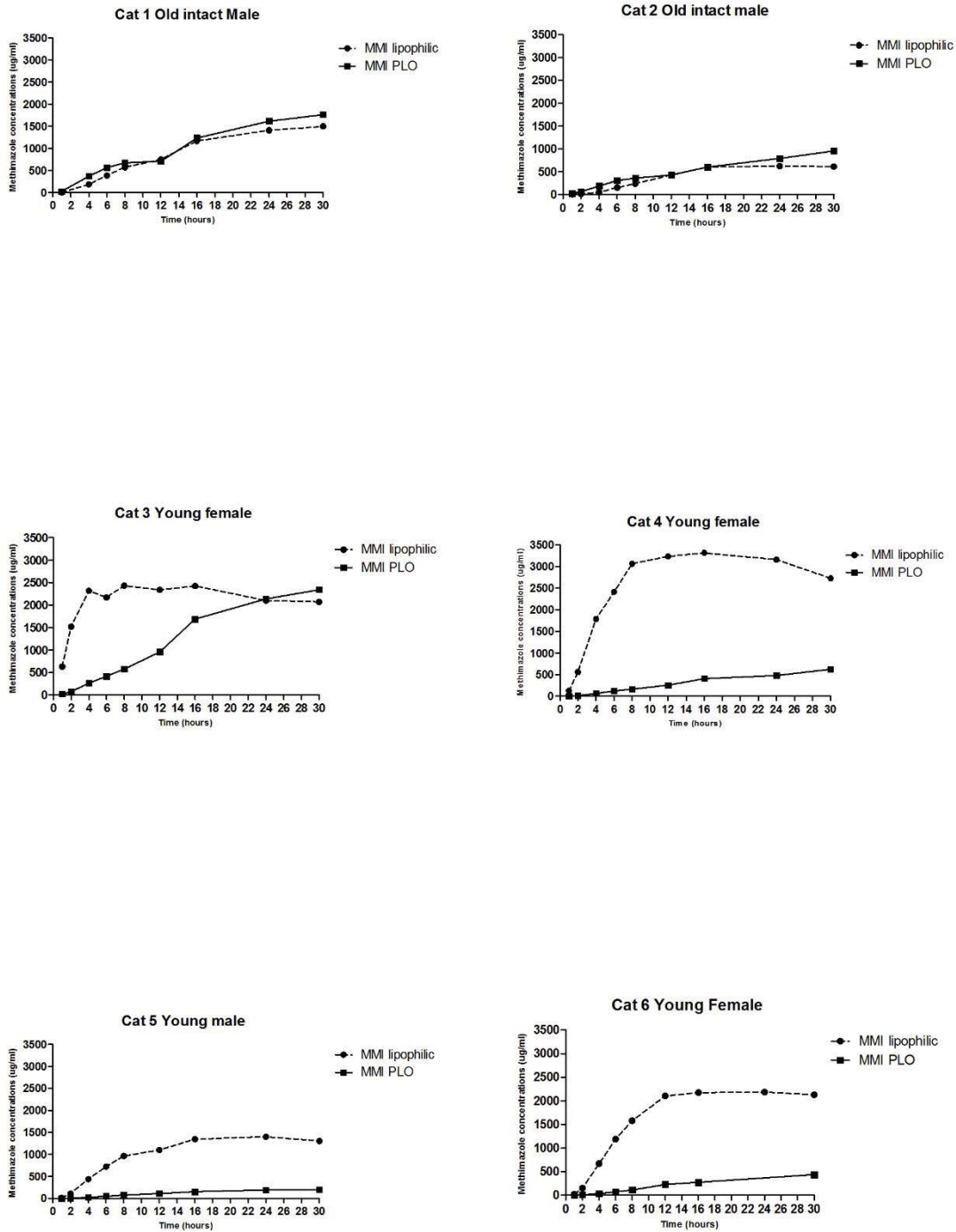
Figure 3: Individual cumulative methimazole (MMI) concentrations in six pairs of cat ears (three males and three females) after the application of 10 mg MMI in Pluronic® lecithin gel (PLO) to the left ear, or 10 mg MMI in a lipophilic vehicle using an *in vitro* Franz cell model.

Cumulative methimazole concentrations over time for young and older cats for each

vehicle is shown in Figure 4.
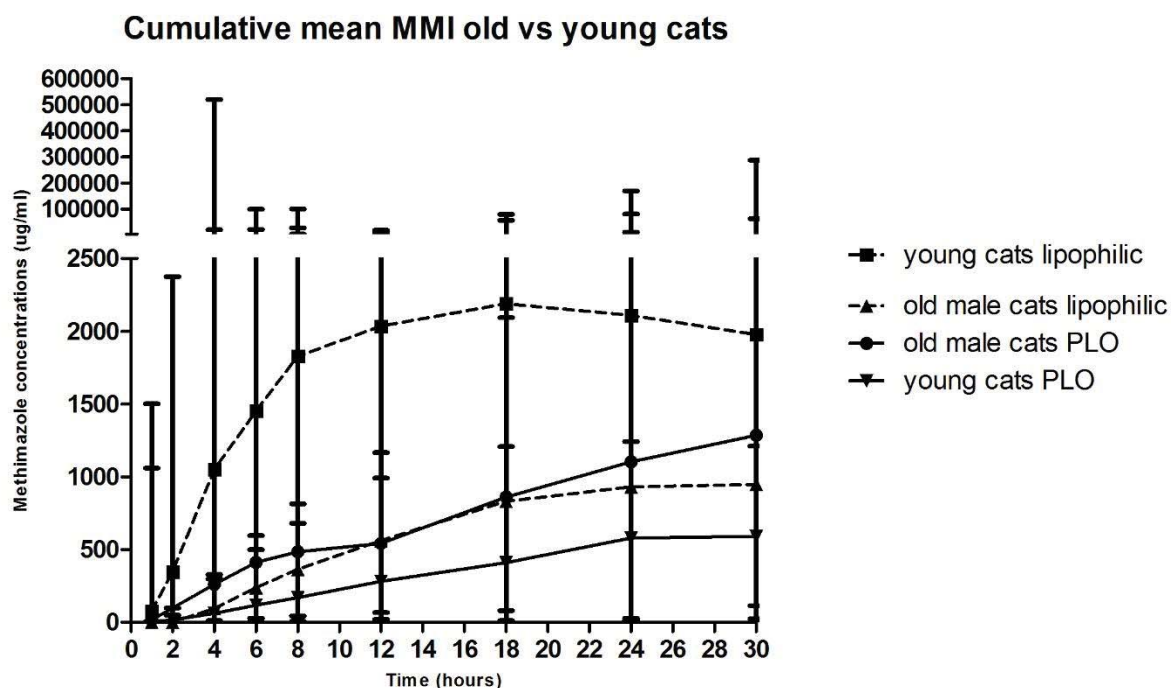
Figure 4: Cumulative methimazole (MMI) concentration after the application of 10 mg of MMI in
Pluronic® lecithin gel (PLO) to the left inner pinna skin, or MMI in a lipophilic vehicle to right inner
pinna skin discs of two older intact male cats and 4 young cats using an *in vitro* Franz cell model. The
error bars represent 95% confidence intervals.

The mean total absorption of methimazole after completion of the experiment is shown

in Table 1. The total mean absorption of methimazole was greater in the lipophilic

vehicle compared to PLO gel, however this value was not significant (p=0.13).

Histology of the ears showed no evidence of significant freezing artefact such as cell

disruption. The mean thickness of the stratum corneum, epidermis and dermis are

shown in Table 2. (Individual cat data is shown in Appendix 1).

Table 1: Mean total absorption of methimazole (MMI) in a lipophilic vehicle and PLO gel after 10 mg was applied to six pairs of ear skin discs of cats using the *in vitro* Franz model. Receptor solution = Phosphate buffered saline with 4% bovine serum albumin.

	MMI in lipophilic vehicle			MMI in PLO gel		
	Mean	Lower CI	Upper CI	Mean	Lower CI	Upper CI
Mean amount of MMI (mg) in receptor solution	5.4	3.1	9.4	2.6	1.02	7
Mean amount of MMI recovered from skin (mg)	0.16	0.06	0.4	0.62	0.21	1.8
Mean absorption of MMI (mg)	5.7[a]	3.5	9.3	4.15	2.38	7.26
Total absorption of MMI as % of 10 mg MMI applied dose)	57%			42%		

[a]p=0.13

Table 2: The mean, standard deviation (SD) and range of thickness (µm) of the inner ear epidermis, stratum corneum (corneum), dermis and number of epidermal cell layers from the left and right ears of six cats (three males and three females) as determined by paraffin histology sections.

	Total mean	SD	Male mean	SD	Female mean	SD	Range
Epidermis	23.49	10.42	17.25	3.42	29.74	2.45	11.00-39.39
Corneum	3.42	2.69	3.98	1.88	2.87	8.52	1.23-11.52
Dermis	410.18	179.18	468.56	24.31	351.80	29.20	222-837
Cell layers	3.64	1.16	3.17	0.37	4.11	0.39	2.00-5.67

DISCUSSION

The two significant outcomes from this study were that: (i) topically-applied methimazole can penetrate through the skin of the inner pinna of the cat; and (ii) the rate and extent of this penetration is formulation dependent. The first outcome was an important result since grooming or licking has been shown to contribute to plasma drug concentrations of topically applied formulations in cattle (Toutain et al., 2012; Sallovitz et al., 2005). Other studies in cats where plasma drug concentrations have been measured after transdermal application of drugs, have not proven that the drug was not ingested (Scherk-Nixon, 1996; Hoffman et al., 2002; Ciribassi et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Bennett et al., 2005; Helms, 2007; MacGregor et al., 2008; Hill et al., 2011; Hill et al., 2014; Miller et al., 2014).

This study has shown that methimazole will cross the skin of the inner pinna of the cat and thus contribute, at least in some proportion to the concentration of active drug in the serum and, hence, the efficacy of the drug.

The second major outcome was that the formulation or vehicle had a significant effect on the transdermal penetration of methimazole in the cat. This finding could be expected from studies in other species, such as the dog and the horse (Mills et al., 2005; Mills et al., 2006; Ahlstrom et al., 2013). The study, for the first time in cats, showed that a novel formulation of methimazole in a lipophilic vehicle can penetrate the ear skin to a greater extent than methimazole in the PLO vehicle. It is already known that both formulations provide effective therapy for cats with hyperthyroidism (Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006; Hill et al., 2011; Boretti et al., 2013).

The aim of the current study was to characterize the percutaneous absorption pharmacokinetics of methimazole in a novel lipophilic vehicle compared to

methimazole in PLO gel on feline ears using a finite dose *in vitro* Franz cell model.

Both the cumulative absorption and flux of methimazole (Figures 1 and 2) in the novel

lipophilic vehicle were significantly greater compared to the PLO gel over the thirty

hour experiment. The cumulative absorption profile (Figure 1) of methimazole in the

PLO gel suggests that absorption of methimazole was obviously not complete over the

thirty hour time period. It is uncertain what specific differences between the two

vehicles affected the transdermal penetration of the methimazole. The major area of

skin that provides resistance to drug penetration through the skin is the stratum corneum

(Mills & Cross, 2006; Lane, 2012; Jepps et al., 2013). One of the roles of the vehicle is

to help the drug permeate the lipid bilayer of the stratum corneum and subcutaneous

tissues (Finnin & Morgan, 1999). Lipophilic drugs (with a log P between -1 to 3) are

considered most suitable for transdermal delivery (Finnin & Morgan, 1999;

Chandrasheka & Shobha Rani, 2008). Methimazole (log P 0.75), may be more suited to

a lipophilic vehicle, thus PLO gel might not be the most suitable vehicle for a lipid

soluble drug (Mills & Cross, 2006). A suitable vehicle will allow the active drug to

remain soluble and prevent the drug precipitating out of solution (Mills & Cross, 2006).

The methimazole in a lipophilic vehicle, was a commercial product (Hyper-T™

Earspot), with proven stability, and may be a more suitable vehicle to aid methimazole

transport through the stratum corneum (Nanjan et al., 2010). The pharmacokinetics of

this lipophilic formulation have been previously described in cats and the lipophilic

formulation has been shown to be an effective therapy of hyperthyroidism in cats

(**Chapters 3 & 4**) (Hill et al., 2011; Hill et al., 2014). Methimazole in the PLO gel has

previously been shown to have problems with precipitation of the drug and

development of a non-homogenous gel texture (Lecuyer et al., 2006). In the current

study, gross precipitation of methimazole in the PLO gel was not evident, and the study

was performed within one week of preparation of the methimazole/PLO gel formulation. However, it is possible that the methimazole was not evenly distributed in the PLO gel which may have accounted for some of the difference in absorption seen in the current study.

Considerable individual variation in the concentration of methimazole was observed between the six cats (Figure 3). The inner pinnal skin from the two older intact male cats was less permeable to the lipophilic formulation of methimazole compared to the younger cats, however the permeability of methimazole between the two vehicles for the intact male cats was comparable (Figure 4). Statistical analysis of the effect of age and neuter status, was not performed due to the small sample size (n=2) and the large confidence intervals. The reason for this trend with intact male cat skin is unclear.

The ear skin of the intact male cats had longer storage at -20°C than the ear skin from the other four cats. However, previous studies have shown that freezing has tended to increase transdermal drug permeability (Ahlstrom et al., 2007) and there were no apparent gross or histological changes, such as cell disruption, that indicated damage due to freezing. The histology of the two intact male cats did show a thickened dermis in one cat, and a slight increase in dermal thickness in the other (Table 2). Similar gender differences in skin thickness have been noted in other species, including humans where males have thicker skin in some regions, compared to females, while female skin generally thins after menopause (Giacomoni et al., 2009). However the major barrier to drug permeation is the stratum corneum, which was not thicker in the two intact male cats. The most likely explanation is individual variation, although further histology on the ears of male cats is warranted. More relevant is that each cat served as its own control when comparing the two formulations, therefore any differences between gender and age would not affect the overall outcomes from the current study and would also be

likely to reflect the differences between the two formulations in the wider cat population. For the PLO formulation, little difference was seen between the intact male cats and the younger cats. The increased permeability of the lipophilic formulation of methimazole in the younger cats may indicate age and neuter interactions, or could be a result of vehicle interactions with the younger cat skin. To the authors' knowledge, skin and gender differences in cats has not been studied. Further research into whether the combination of the age, gender and neuter status and the type of vehicle affects percutaneous drug absorption would be recommended.

Histology of the inner pinnal skin did show considerable intra-subject variation (Table 2), however the differences in skin thickness did not account for the variability in drug perfusion. Only one other study has compared the thickness of the epidermis and stratum corneum of the ear (Monteiro-Riviere et al., 1990). In the study by Monteiro-Riviere et al, the stratum corneum thickness (3.94 μm ± 0.44) were comparable to the current study, however the epidermal (14.71 μm ± 1.36) and number of cell layers (1.39 ± 1.01) were different. The differences between the two studies may be due to the methods employed, as it is unknown whether the inner or external epidermis and stratum corneum of the ear was examined, and gender of the experimental cats was not provided (Monteiro-Riviere et al., 1990).

From the current study, the differences in inner pinnal skin thickness are likely to be individual variation between cats and possibly to the different planes of section between different areas within the same section. In the current study, blood flow could not account for the differences in drug perfusion, therefore further research into other skin factors, such as collagen in the dermis or skin appendages is recommended.

There were limitations to the model used in this study, especially the absence of blood circulation under the skin, such as would occur *in vivo*. However, the aim of the study was to determine if active drug actually penetrated skin of the inner pinna, which was confirmed, and to compare kinetics of the two formulations, which would have been difficult *in vivo* due to possible variations in blood flow and the additional difficulty of measuring very low serum concentrations of methimazole. Furthermore, the reservoir solution was not entirely removed at each time point, therefore sink conditions applied initially, with a low concentration of drug in the receptor fluid, which would continue to allow passive drug diffusion (Mills et al., 2004; Mills et al., 2005; Mills et al., 2006). Over time, methimazole concentrations increased in the receptor fluid, which may have prevented further diffusion of methimazole across the skin membrane, and decreased the total amount of methimazole absorbed. Therefore the results from our study may underestimate absorption.

The mean total absorption of methimazole in the lipophilic vehicle (57%) and methimazole in the PLO gel (42%) was relatively low and guidelines published for dermal drug experiments indicate that 90–110% of total drug applied should be recovered at the end of the experiment (OECD, 2004a,b). Low recovery may be due to incomplete application of the finite dose, loss of drug to the experimental equipment, evaporation of drug from the skin, unlabelled test preparations, skin metabolism or degradation, or insufficiently high analytical LODs/LOQs, in particular where non-labelling analytical methods are applied (OECD, 2004a,b). In the current study, the unabsorbed dose of methimazole was not accounted for, which includes the methimazole that remained on the skin, as well as any drug bound to the Franz cells. The unabsorbed methimazole that remained on the skin probably reflects conditions *in*

vivo, therefore in the current study, the meant total absorption, although relatively low, also is likely to reflect *in vivo* conditions.

An obvious weakness of the current study is the lack of replicates, and the large intra-subject variability is reflected in the large standard deviations. Multiple skin replicates from each ear were not possible in this experiment as only one 1.13 cm^2 skin disc could be obtained from each cat ear. To limit inter-subject variability, skin from the left and right ear of each cat was used to apply the different vehicle applications, so each cat served as its own control. Furthermore, histology of the ear skin was also performed to determine physical causes of intra-subject variability.

Another limitation to the study was the frozen cat skin. A decision was made to freeze cat ears since the ears were collected opportunistically as cats were euthanased by a local pound and this procedure was acceptable to the Animal Ethics Committee. Freezing skin can affect the permeation of active drug, however the permeation affects has been shown to be a relatively predictable change over time in the dog and sheep (Ahlstrom et al., 2007; Bayldon, 2012). In summary, short term freezing has a small but predictable effect to increase the permeability of some drugs, although the amount of time over which the ears were frozen for the current study would have been unlikely to have affected drug permeability. In addition, histology was performed to determine if any freezing artefact could be seen and whether there were any differences between cats or any skin pathology that may influence drug penetration. More importantly, each animal acted as its own control, so any differences due to freezing would not have affected the relative difference in penetration between the two formulations.

A further limitation of the study was the high inter-individual variability. This limitation could be expected and, again, reflects the variability in the cat population. However,

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each cat served as its own control when comparing the two formulations, with statistical analysis confirming significant penetration, and possible differences between each formulation which might have a clinical impact.

The results of the current study have shown that methimazole is absorbed across the inner pinnal skin of cats using an *in vitro* model. The commercial lipophilic formulation had more complete absorption across the inner pinna skin than methimazole in PLO gel.

CONFLICT OF INTEREST

Kate Hill and J. Paul Chambers receive some royalties for the sales of Hyper-T™ Earspot in New Zealand.

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Appendix 1

Table 1: Thickness (μm) of the inner ear epidermis, stratum corneum (SC), dermis and number of epidermal cell layers from the left and right ear of six cats as determined by paraffin histology sections. The mean and standard deviation (SD) of three replicates is provided. M = Male, FS = female spayed, MC = male castrated.

Cat	L or R	Epi-dermis (μm)	SD	SC (μm)	SD	Cell layers	SD	Dermis (μm)	SD
1M	L	12.92	1.06	1.23	0.23	3.33	0.58	374.26	45.51
	R	12.64	3.81	1.77	0.95	3.33	0.58	399.05	13.62
2M	L	11	1.73	3.42	3.52	3.00	1.00	692.53	54.49
	R	17.55	4.66	11.52	4.92	3.33	0.58	837.13	72.65
3FS	L	36.87	6.61	3.18	0.77	5.67	0.58	380.01	10.42
	R	38.55	2.64	3.5	1.59	5.67	0.58	425.55	17.1
4FS	L	22.24	0.81	2.64	1.11	3.33	0.58	316.17	77.24
	R	39.39	6.59	3.21	21.91	5.00	1.00	382.75	39.85
5MC	L	26.3	10.66	3.71	1.09	3.00	1.00	222.69	9.46
	R	23.05	3.66	2.24	0.65	3.00	0	285.68	44.35
6FS	L	27.42	3.59	3.25	1.12	3.00	0	251	72.74
	R	13.99	1.76	1.42	0.71	2.00	0	355.35	67.06
Total Mean		23.49	10.42	3.42	2.69	3.64	1.16	410.18	179.18
Mean male		17.25	3.42	3.98	1.88	3.17	0.37	468.56	24.31
Mean female		29.74	2.45	2.87	8.52	4.11	0.39	351.80	29.20

Chapter 6

Regional variations in percutaneous absorption of methimazole: an *in vitro* study on cat skin

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PREFACE

The study in **Chapter 5** revealed that methimazole in two different vehicles (PLO gel and a lipophilic formulation) could be absorbed across the ear skin of cats using an *in vitro* model. The commercial lipophilic formulation had more complete absorption across the pinnal skin than methimazole in the PLO gel. The inner pinna has been the preferred site for the application of methimazole for transdermal absorption, as it is relatively hairless and has limited access to self-grooming (Hoffmann et al., 2003) however, the preferred skin region to apply methimazole for transdermal absorption in cats has not been studied. Therefore, the study in this chapter describes work that characterises the percutaneous absorption pharmacokinetics of methimazole in a novel lipophilic vehicle applied to various skin regions in an *in vitro* Franz cell model.

Part of this chapter was accepted to the *Journal of Veterinary Pharmacology and Therapeutics* and is formatted for the style of that journal.

ABSTRACT

The use of transdermal gel medications in cats has become popular in veterinary medicine due to the ease of administration compared to oral medication. The research to support systemic absorption of drugs after transdermal gel administration and the preferred skin region to apply these drugs in cats is limited. The aim of the current study was to characterize the effect of different skin regions on the percutaneous absorption pharmacokinetics of a commercially available transdermal methimazole after a finite dose was applied to feline skin *in vitro*. A commercial formulation of methimazole (10 mg) was applied to four skin regions (the inner stratum corneum of the ear, groin, neck and thorax regions) from six cats. The receptor medium was sampled up to 36 hours post application and methimazole concentrations were measured using high performance liquid chromatography. Histology of all skin regions was undertaken as small differences in the thickness of skin may have contributed to inter-individual differences in methimazole absorption between the six cats. Methimazole was absorbed more completely across the pinnal skin, compared to the skin of the groin, neck and thorax ($p < 0.001$), which justifies application to the pinna to maximize efficacy and also to minimize the effects of grooming.

Keywords: transdermal, methimazole, cats, *in vitro*

INTRODUCTION

The function of the skin is to prevent excessive water loss and to provide a barrier to toxins, allergens and drug permeation. The stratum corneum (the non-viable epidermis) is the major layer of the skin that prevents molecules penetrating through into the dermis, which is more permeable to polar compounds such as water (Mills & Cross, 2006b; Benson 2012). Despite the skin being an effective barrier to drug uptake, transdermal drug therapy has become popular in cats since the early 2000s with many formulations now available for systemic and local effects (Riviere & Papich, 2001; Marks & Taboada, 2003; Boothe, 2006).

There are several factors that affect the transdermal delivery of drugs, including their size (< 500 Da), availability of hydrogen bonds, lipophilicity and having a low melting point (Mills & Cross, 2006b). Importantly, the transdermal absorption of drugs can vary greatly depending on the region of application on the body. Studies into the differential regional absorption of transdermal drugs in humans date back to the 1960s, and show that the highest absorption of drugs is from the scrotal area, and lowest in the heel (Feldmann & Maibach, 1967; Moe & Armstrong, 1986; Ebihara et al., 1993). The order of regional variation in skin barrier function in humans is genitals > head and neck > trunk > arm and leg (Feldmann & Maibach, 1967). Similarly, the region of application has been reported to affect the systemic concentrations of topically-applied drugs in the horse and dog. For example, in the horse, methylsalicylate was absorbed through skin of the leg region to a greater extent than that of the thorax and groin, while the converse was true for fentanyl (Mills & Cross, 2006a; Mills & Cross, 2007a; Mills & Cross, 2007b). In dogs, hydrocortisone and testosterone in a 50% ethanol vehicle had a nearly two fold greater flux through neck skin compared to inguinal skin (Mills et al., 2005; Mills et al., 2006) and fentanyl was absorbed faster through groin skin than neck skin

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(Mills et al., 2004b). To the authors' knowledge, no studies on the regional application of transdermal drugs to feline skin have been performed.

In cats, the inner pinna is the most common site for transdermal drug therapy for ointments and gels as opposed to liquid spot-ons and patches. The inner pinna is relatively hairless, has a thin stratum corneum and has limited exposure to self-grooming (Monteiro-Riviere et al., 1990; Hoffmann et al., 2003). The skin of cats is thinner than dogs, with an average thickness of 0.4–2.0 mm compared to 2.6–5.5 mm in dogs (Strickland & Calhoun, 1963; Young et al., 2002). Cat skin is thickest in the dorsal regions and proximal limbs, and thinnest on the ventrum and distal limbs (Strickland & Calhoun, 1963). The stratum corneum and epidermis of cat skin is also thinner than dog skin, with the abdomen and ear having the thinnest stratum corneum (Monteiro-Riviere et al., 1990). The differences in skin anatomy between species make extrapolation of transdermal drug absorption kinetics impractical (Mills & Cross, 2007a), and indeed, cats have lower transdermal bioavailability of fentanyl, compared with dogs (Kyles et al., 1996; Lee et al., 2000; Murrell et al., 2007).

The aim of the present study was to compare the absorption of a commercially available transdermal methimazole formulation through feline skin collected from different regions of the body. The outcomes from this *in vitro* study will determine if the active drug is likely to penetrate skin, and therefore be systemically active, when applied to different body regions and also if the pinna is the most suitable site to apply topical formulations in the cat.

MATERIALS AND METHODS

Animals

Six domestic short haired cats (five female and one castrated male) at a local pound were euthanased by an intravenous injection of sodium pentobarbital. Full thickness skin from the groin (defined as caudal to the last nipple), thorax (defined as the dorso-ventral line intersecting the caudal angle of the scapula; dorsal border: transverse processes of the thoracic vertebrae; caudo-ventral border: an arc joining the dorsal border from the last rib, to the cranial border at the level of the costochondral junction), neck (defined as caudal to wing of atlas and cranial to scapula, with dorso-ventral borders drawing a line between atlas and scapular) and ears were harvested soon after death.

The ears and skin were frozen at -20°C for five months until required. This experimental protocol was approved by the Animal Ethics Committee of the University of Queensland (approval number SVS/494/12).

Ear skin was defrosted and transected with gentle blunt dissection to separate the ventral (inner surface) from the dorsal aspect of the ear. Subcutaneous tissue and cartilage tissue were removed. The skin and transected ear skin was rinsed with tap water to remove any blood and then dried gently with a gauze swab. One circular section of skin measuring 2 cm^2 diameter was cut from each ear cut using a round biopsy punch, and two circular sections measuring 2 cm^2 diameter were cut from the skin of the neck, groin and thorax.

Histology

Samples of the ear skin surrounding the hole left from the biopsy punch were trimmed and samples of remaining thoracic, neck and groin skin were trimmed and fixed in 10%

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buffered formalin for 24–48h, processed and embedded into paraffin blocks. Analysis was performed as previously described in **Chapter 5** and **Appendix A**. The inner pinna of the left and right ears of each cat were examined as well as samples from the groin, neck and thorax.

In vitro skin penetration studies

Skin was mounted onto Franz-type diffusion cells with the stratum corneum uppermost as previously described in **Chapter 5** and **Appendix A** (Mills et al., 2005; Hill et al., 2015). The methimazole in a lipophilic formulation (Hyper-T™ Earspot, Bayer NZ Ltd, Auckland New Zealand) was applied to the upper surface of the skin at a finite dose (10 mg). Therefore the cumulative methimazole concentration and absorption curves were analysed for finite dose conditions. A 200 µL sample of reservoir solution was removed at 1, 2, 4, 6, 8, 12, 18, 24, 30 and 36 h and was replaced by 200 µL of fresh solution into the sampling port. The aliquots (200 µL) were frozen at -20°C until subsequent analysis, within two months of sampling.

At the end of the experiment, the Franz cells were dismantled, the skin removed, and patted dry with gauze swabs. The skin exposed to the formulation containing methimazole was excised and macerated with scissors. Macerated samples were then placed in pre-weighed vials, and re-weighed. Samples were labelled and frozen at -20°C until required for further analysis within two months of sampling.

The methods for high performance liquid chromatography (HPLC) analysis of methimazole from serum and extraction of methimazole from the skin have been previously described in **Chapter 5** and **Appendix A** (Hill et al., 2011; Hill et al., 2015).

Data and statistical analyses

The concentration of methimazole in the receptor fluid at each collection time was measured. To calculate the true methimazole concentration at each time point, accounting for the dilution factor of 200 µL being removed from the 3.5 mL chamber, a correction factor was applied.

The flux (J) was calculated using the formula $J = Q/(At)$ (Franz et al., 2009). The cumulative amount of methimazole that had permeated the skin to the receptor fluid was calculated for each replicate at each sampling time point. The geometric mean with 95% confidence intervals was calculated for each region. Differences in the rates of methimazole absorption and flux by region were compared using linear regression models with region and time as fixed effects; an interaction between time and region was also tested. Analyses were conducted in Stata version 12 (Statacorp LP, College Station, Texas) and a value of $p < 0.05$ was considered significant.

The mean total absorption of methimazole was calculated from the addition of the methimazole concentration in the receptor fluid (absorbed dose of drug) to the methimazole recovered from the skin samples (absorbable dose of drug). Data is shown as geometric mean and 95% confidence intervals.

A one-way ANOVA with a post-hoc Bonferroni multiple-comparisons test was used to compare the mean absorption of methimazole in the skin and receptor in each region, and the difference between the thickness of the stratum corneum and the epidermis in each region. Analyses were conducted in Stata version 12 (Statacorp LP, College Station, Texas) and a value of $p < 0.05$ was considered significant.

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RESULTS

Figure 1 shows the cumulative methimazole concentrations, and Figure 2 shows the rate of absorption (flux) of methimazole over the 36 h time course. A significant effect of skin region was found for both cumulative methimazole concentrations ($p<0.001$) and flux ($p<0.001$).

Figure 1: Cumulative methimazole (MMI) with vertical error bars representing 95% confidence intervals in six cats after application of 10 mg of methimazole to skin of the ear, neck, groin or thorax over 36 hours. Each data point represents the geometric mean of two replicates from six cats. The cumulative amount of methimazole per µg/mL is on the y axis. There was a significant difference ($p<0.001$) of region.

Rate of Absorption (Flux)

Figure 2: Mean methimazole absorption (flux) (MMI) with vertical error bars representing 95% confidence intervals in six cats after application of 10 mg of methimazole to skin of the ear, neck, groin or thorax using a concentrations over 36 hours. Each data point represents the geometric mean of two replicates from six cats. There was a significant difference ($p<0.001$) of region.

The mean total absorption of methimazole at the end of the experiment is shown in Table 1. There was an effect of region on the mean total absorption of methimazole in the receptor ($p=0.044$) but not for recovery from the skin ($p=0.774$) at the completion of the experiment.

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Table 1: The mean total absorption of methimazole (MMI) in a lipophilic vehicle applied to four regions of skin from six cats using the *in vitro* Franz model. Each data point represents the mean of six cats, each region with two replicates. The left and right ears have been combined. Receptor solution = Phosphate buffered saline with 4% bovine serum albumin. Means with different superscripts within row are different ($p<0.05$).

	Ear			Neck			Groin			Thorax		
	Mean	Lower CI	Upper CI	Mean	Lower CI	Upper CI	Mean	Lower CI	Upper CI	Mean	Lower CI	Upper CI
Mean amount of MMI in receptor solution (mg)	5.4[a]	4.7	6.2	3.9	3.0	5.1	3.7	2.7	5.2	3.4[b]	2.6	4.5
Mean amount of MMI recovered from skin (mg)	0.2	0.1	0.6	0.2	0.1	0.4	0.3	0.1	0.6	0.3	0.2	0.6
Mean absorption of methimazole (mg)	5.9	5.1	6.8	4.0	2.9	5.6	4.3	3.4	5.5	3.9	3.2	4.8
Total absorption (% of 10 mg methimazole applied dose)		59%			40%			43%			39%	

The thickness of the various areas of skin is shown in Table 2. Histological analysis of the ears showed minimal evidence of significant freezing artefact such as cell disruption or tissue vacuolation (Figure 3). The mean thickness of the inner ear, cartilage and outer ear is shown in Table 2. A significant difference between skin region was found for the epidermis (p<0.001). The epidermis was thinner in the groin (p=0.001) and thorax samples (p=0.006) compared to the ear samples. There was no difference in skin region for the thickness of the stratum corneum (p=0.601).

Table 2: Thickness (µm) of the epidermis, stratum corneum, and number of epidermal cell layers from various regions of six cats as determined by paraffin histology sections. The mean and standard deviation (SD) of three replicates is provided. Means with different superscripts within row are different (p<0.05).

	Ear (n=11)	SD	Groin (n=3)	SD	Neck (n=3)	SD	Thorax (n=6)	SD
Epidermis (μm)	14.65[a]	2.39	6.29[b]	0.61	12.11[ab]	3.68	9.06[b]	3.51
Corneum (μm)	10.41	4.36	5.6	1.19	9.52	3.66	10.47	9.01
Cell layers	2.22	0.28	1.33	0	2	0.33	1.67	0.3
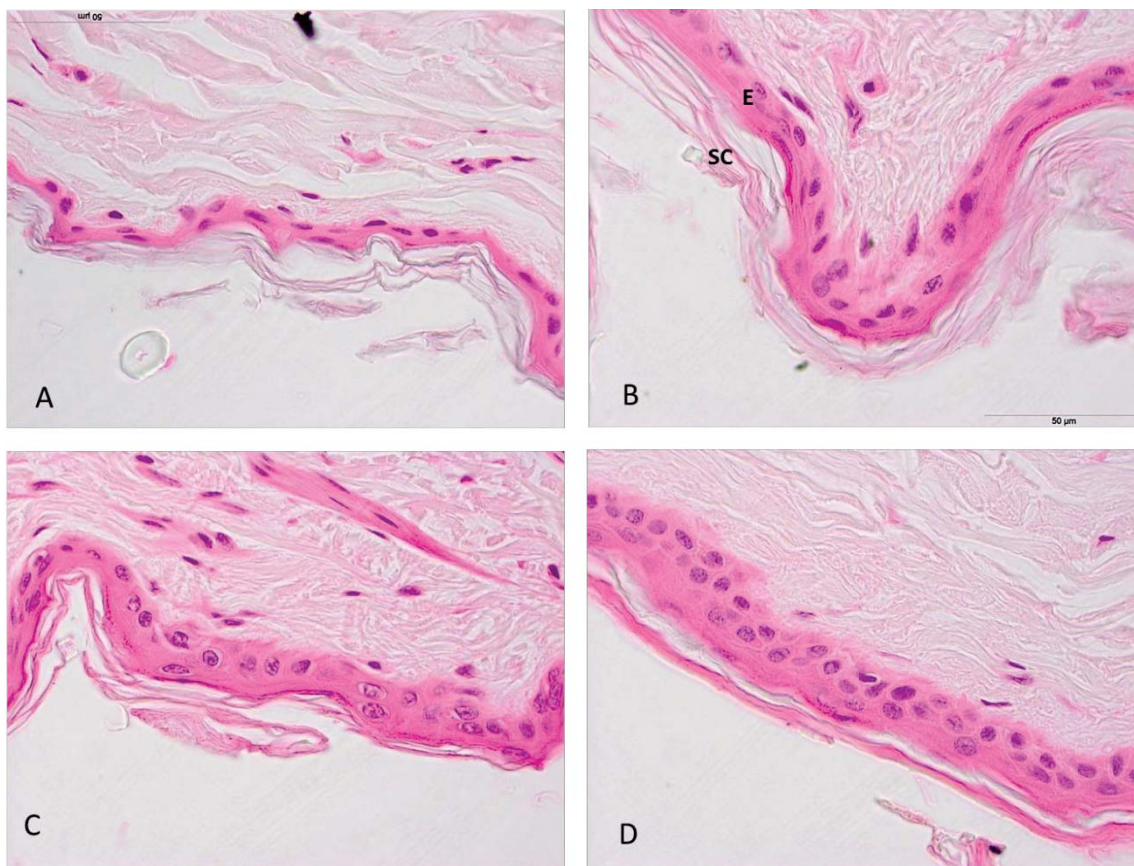
Figure 3: Haematoxylin and eosin (H&E) (1000x) stained images of the groin (A), neck (B), thorax (C) and left ear inner epidermis (D) of cats in the study on the effect of region on the absorption of transdermal methimazole. The stratum corneum layer (SC) and epidermal (E) layers are labelled in B. A significant difference in region was found for the epidermis ($p<0.001$). The epidermis was thinner for the groin compared to the ear ($p=0.001$) and the ear and thorax ($p=0.006$). There was no difference in skin region for the thickness of the stratum corneum ($p=0.601$).

DISCUSSION

Transdermal drug therapy has become popular in cats since the early 2000s with many compounding pharmacies formulating drugs into gels for systemic absorption (Marks & Taboada, 2003; Boothe, 2006). In cats, the inner pinna is the most common site for transdermal drug therapy for ointments and gels and the current study has confirmed that the ear, compared to other skin regions, has significantly greater absorption of methimazole when applied in a lipophilic vehicle.

This study used a finite dose of transdermal methimazole developed for application to the pinna (Nanjan et al., 2010). The absorption of methimazole in the ear region (Figure 1) reached a steady-state, whereas absorption in the thorax, neck and groin was incomplete 36h after application. The three skin regions were chosen for this study based on previous studies using dogs based on likely regions where a topical formulation may be applied (Mills et al., 2004a; Mills et al., 2004b; Mills et al., 2005; Mills et al., 2006). In cats, the thorax and neck skin region would not be very practical areas to apply a transdermal gel, as self-grooming access and the potential for owner contamination with the drug would be high, unless the drug was under a plastic coating. The groin area would also be accessible to self-grooming, but the potential for owner contamination with the drug would be lower. The current study has shown that the ear was the preferred area for gel formulations developed for topical application, with the fastest absorption of transdermal methimazole and the lowest chance of the cat (or owner) rubbing the ointment from the ear before the drug is absorbed.

It was unclear why methimazole was absorbed more completely through the ear in the current study. Previous studies in dogs, have shown lipophilic drugs such as fentanyl are absorbed best from the groin area (Mills et al., 2004b). However, in the current study, the increased absorption through the ear could not be related to blood flow, since this

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was an *in vitro* study using harvested tissue from euthanised animals. *In vivo* blood flow to the ear of the cat is generally higher than to the humero-scapular and the thoraco-lumbar junction areas, but similar to the ventral abdominal skin near the umbilicus (Monteiro-Riviere et al., 1990).

In the current study, the thickness of the stratum corneum was similar for each region and (Table 2), although the epidermal skin from the groin and thorax was thinner (Table 2). The epidermal thickness did not correlate with methimazole absorption (Table 1), an expected finding, as it is the stratum corneum (the non-viable epidermis) that is the major layer of the skin that prevents molecules penetrating through into the dermis, not the epidermis (Mills & Cross, 2006b; Benson 2012). One notable difference in the skin from the ear was the density of hair follicles, which was lowest in the pinna skin. Skin appendages are possible routes of transport for some drugs (Tur et al., 1991; Hueber et al., 1994; Grice et al., 2010), but the physicochemical properties of methimazole appear to favour passage through the relatively hairless pinnal region, although further studies are required to determine if other factors also contribute. Irrespective, the relatively higher blood flow to the pinna would be expected to further support application of methimazole formulations to the pinna of cats *in vivo*.

Considerable individual variation in the absorption of methimazole was observed between the six cats (Figure 1 & 2). High intra and inter-individual variability is expected in *in vitro* transdermal trials and reflects the variability in the cat population (Finnin et al., 2012). Skin from all six cats was frozen for five months, since skin was collected opportunistically as cats were euthanased by a local pound and this was acceptable to the Animal Ethics Committee. Previous studies have shown that short term freezing has tended to increase transdermal drug permeability with a small but predictable effect although the amount of time over which the skin was frozen for the

current study would have been unlikely to have affected drug permeability (Ahlstrom et al., 2007). If freezing the skin did effect permeability, the increase in permeability would be expected to have a similar effect on the different skin regions. Similarly, there were no apparent gross or histological changes, such as cell disruption, that indicated damage due to freezing, but any specific effects on transdermal penetration of drugs would be expected to be consistent between the sites.

There were limitations to this study, including the absence of blood circulation under the skin, such as would occur *in vivo*. However, the aim of the study was to determine if the region of application affected the kinetics of transdermal methimazole absorption, which would have been difficult *in vivo* due to possible variations in blood flow and the additional difficulty of measuring very low serum concentrations of methimazole. Furthermore, the reservoir solution was not entirely removed at each time point, therefore sink conditions applied initially, with a low concentration of drug in the receptor fluid, which would continue to allow passive drug diffusion (Mills et al., 2004b; Mills et al., 2005; Mills et al., 2006). Over time, methimazole concentrations increased in the receptor fluid, which may have prevented further diffusion of methimazole across the skin membrane, and decreased the total amount of methimazole absorbed. Therefore the results from this study may underestimate absorption.

The mean total absorption of methimazole from the ear (59%), neck (40%), groin (43%) and thorax (39%) was relatively low and guidelines published for dermal drug experiments indicate that 90–110% of total drug applied should be recovered at the end of the experiment (OECD, 2004). Low recovery may be due to incomplete application of the finite dose, loss of drug to the experimental equipment, evaporation of drug from the skin, unlabelled test preparations, skin metabolism or degradation, or insufficiently high analytical LODs/LOQs, in particular where non-labelling analytical methods are

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applied (OECD, 2004). In the current study, the unabsorbed dose of methimazole was not accounted for, which includes the methimazole that remained on the skin, as well as any drug bound to the Franz cells and experimental equipment. The most likely reason for low recovery of methimazole in this study, was the unabsorbed methimazole that remained on the skin, an event which, in hindsight, probably reflects conditions *in vivo*.

In vitro, methimazole in a commercial lipophilic formulation is absorbed more completely when applied to the ear skin of cats compared to skin from the groin, neck and thorax regions. The application of transdermal methimazole to the ear of cats would be the preferred application area, however further *in vivo* studies are recommended to confirm these findings.

CONFLICT OF INTEREST

Kate Hill and J. Paul Chambers receive some royalties for the sales of Hyper-T™

Earspot in New Zealand.

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Chapter 7

Trans-pinnal movement of methimazole: an *in vitro* study showing that methimazole can cross from the inner to outer pinna of cats.

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PREFACE

The study in **Chapter 5** revealed that methimazole in a lipophilic vehicle could be absorbed across the pinnal skin of cats using an *in vitro* model. The study in **Chapter 6** revealed that the best skin region to apply methimazole in a lipophilic vehicle was the skin of the inner ear. However, it is unknown whether methimazole in a lipophilic vehicle applied to the internal pinna (non-haired region) will cross through the ear to the external (haired) pinna. Therefore, **Chapter 7** investigates whether methimazole in a novel lipophilic vehicle applied to the inner pinna will cross to the external pinna in an *in vitro* Franz cell model.

This chapter was published in the *Journal of Feline Medicine and Surgery* and is formatted in the style of that journal.

ABSTRACT

Objectives

To determine if methimazole applied in a transdermal formulation to the internal pinna will cross to the external pinna in an *in vitro* Franz cell model.

Methods

The ears from six cats were harvested soon after death. Whole ears were mounted onto Franz-type diffusion cells with the stratum corneum of the inner pinna uppermost. A commercial transdermal preparation containing methimazole (0.1 mL/10 mg) was applied to the inner pinna. At 1, 2, 4, 6, 8, 12, 18, 24 and 30 h, a 200 µL sample of reservoir solution was removed to determine the methimazole concentration by high performance liquid chromatography. The ears were then dissected, separating the internal pinna from the cartilage and the external pinna, before the methimazole concentration was measured at each site. The thickness of the different regions of the ear was measured on paraffin histology sections.

Results

Mean ± SD methimazole concentrations at 30 h for the right and left ear, respectively, were inner ear (1.25 ± 0.53 mg/g, 0.39 ± 0.26 mg/g), cartilage (1.36 ± 0.47 mg/g, 0.33 ± 0.20 mg/g) and outer ear (1.0 ± 0.32 mg/g, 0.33 ± 0.14 mg/g). There was a difference between the left and right ears ($p<0.001$). Minimal methimazole concentrations were detected in the receptor fluid. The mean methimazole concentration absorbed by the skin after application of 10 mg was, for the right ear, 3.65 ± 1.27 mg/g and, for the left, 1.08 ± 0.27 mg/g. There was no correlation between methimazole concentrations and thickness of each region of the ear.

Conclusions and clinical relevance

Methimazole in a lipophilic vehicle applied to the inner pinna will penetrate to the outer pinna of cats in an *in vitro* model, a finding which may have safety implications for humans associated with cats treated with transdermal methimazole. Substantial inter-individual variation was found. Further research is required in the area of transdermal penetration of drugs in cats.

Keywords: feline, transdermal, methimazole, cats, *in vitro*

INTRODUCTION

Transdermal drug therapy has become popular in cats since the early 2000s with many compounding pharmacies formulating drugs into gels for topical application and systemic absorption and action (Marks & Taboada, 2003; Boothe, 2006). Despite a recent survey in the United Kingdom reporting that 62% of cat owners could administer a pill *per os* to their cat twice a day without a problem, many cat owners find it difficult to medicate their cats thus transdermal drug therapy for cats is proving a popular alternative to oral medication (Marks & Taboada, 2003; Boothe, 2006; Caney, 2013). In cats the inner pinna is the most common site of application for transdermal drug therapy for ointments and gels as opposed to liquid spot-ons and patches. The inner pinna is relatively hairless, has a thin stratum corneum and has limited exposure to self-grooming (Monteiro-Riviere et al., 1990; Hoffmann et al., 2003).

To date, the drug that has been most extensively studied in cats for transdermal absorption is methimazole (United States Adopted Name [USAN]; thiamazole International Nonproprietary Name [INN]) (Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006; Hill et al., 2011; Boretti et al., 2013; Boretti et al., 2014). The transdermal route provides a good alternative to the oral administration of methimazole for cats with hyperthyroidism, showing both short and long term efficacy (Hoffmann et al., 2003; Sartor et al., 2004; Lecuyer et al., 2006; Hill et al., 2011; Boretti et al., 2013; Boretti et al., 2014). Methimazole is also suitable for topical therapy, since its chemical structure facilitates transdermal penetration (low molecular weight of 144 Da, logP 0.75 and a melting point of 144–147 °C, dose rate less than 20 mg per day), and the efficacy can be assessed easily, as a biological endpoint can be measured (serum thyroxine concentrations and clinical signs) (Finnin & Morgan, 1999). Currently there are no studies in cats that describe the kinetics of methimazole permeation across the skin.

One concern when administering methimazole is that the drug is teratogenic to the human foetus (Clementi et al., 2010; Dechra, 2010; Bayer, 2013). Precautions, such as wearing gloves or finger cots are indicated for people when administering transdermal formulations as the drug can also cross human skin. Washing hands with soap is recommended after use of both oral and transdermal formulations. Recommendations when administering methimazole in any form, are that pregnant women need to wear gloves when handling methimazole, and women of child bearing age should wear gloves when cleaning the litterbox of cats treated with methimazole (Dechra, 2010; Bayer, 2013). While it is obvious that the transdermal formulation of methimazole which remains on the inner pinna should be treated with the same caution as the initial administration, it has not been considered whether the methimazole applied to the inner pinna can pass through the ear and be within the skin of the outer pinna.

Transdermal penetration kinetics can be studied using *in vitro* models such as the Franz cell (Figure 1), and *in vitro* studies correlate well with the *in vivo* situation (Franz, 1975; Franz et al., 2009). The aim of the current study was to determine if the percutaneous application of a commercial transdermal methimazole (currently available in New Zealand) in a lipophilic vehicle, applied to the internal pinna (non-haired region) will cross to the external (haired) pinna of the ear in an *in vitro* Franz type cell model. Throughout this manuscript, the term methimazole is used, the most common term used in the veterinary literature and as stated in the United States Adopted Name (USAN), the Recommended International Nonproprietary Name (rINN) is thiamazole.

MATERIALS AND METHODS

Animals

Six domestic short haired cats (four female, one male neutered, one intact male) at a local pound were euthanased by an intravenous injection of sodium pentobarbital and the pinna harvested soon after death. The ears were frozen at -20°C for three days. This study protocol was approved by the Animal Ethics Committee of the University of Queensland (approval number SVS/494/12).

Whole feline ears were used for the study. The ear was rinsed with tap water to remove any adherent blood and dried gently with a gauze swab. One circular section measuring 2 cm^2 was cut from the same site in each ear using a round biopsy punch.

Histology

Samples of the ear skin surrounding the hole left from the biopsy punch were trimmed to determine thickness of the epidermis, dermis and cartilage and fixed in 10% buffered formalin for 24–48 h, processed and embedded into paraffin blocks. Haematoxylin and eosin (H&E) staining was performed on 3 μm sections of tissue cut from the formalin-fixed, paraffin-embedded (FFPE) blocks which were then examined using light microscopy.

Histological analysis was performed using a Nikon Eclipse Ni microscope. Histological images were captured using NIS Elements software (Nikon Instruments Inc., United States) and analysed using Image J software (Schneider et al., 2012). Briefly, for each sample, the inner and outer stratum corneum, inner and outer epidermis, number of nucleated epidermal cell layers, inner and outer dermis and pinnal cartilage were measured or counted at three randomly selected locations and averaged as previously

described (Monteiro-Riviere et al., 1990). Both left and right ears from each cat were examined.

In vitro skin penetration studies

Whole ears were mounted onto Franz-type diffusion cells (Figure 1) with the ventral inner pinna of the stratum corneum uppermost as previously described (Mills et al., 2005) (**Chapters 5** and **6** and Appendix A). The surface area of inner ear skin exposed to drug in the diffusion cells was 1.13 cm^2. The dermal chamber was filled with 3.5 mL of a reservoir solution suitable for a lipophilic drug of phosphate-buffered isotonic saline (sodium chloride 8.0 g/L, potassium chloride 0.2 g/L, disodium hydrogen phosphate 1.15 g/L, potassium dihydrogen phosphate 0.2 g/L) (PBS) (MP Biomedicals, Sydney, Australia), with 4% bovine serum albumin (BSA)(Sartorelli et al., 2000), pH 7.4±0.1 and the donor compartment left open to ambient laboratory conditions. All cells were mounted in a diffusion apparatus with the dermal bathing solution being magnetically stirred. The temperature of the skin in the diffusion cell was approximately 32°C. After mounting, the upper surface (inner pinna) of the skin was hydrated for 1 h with sodium phosphate buffer (0.1 M) and the system was allowed to equilibrate. The buffer solution was aspirated from the surface of the ear skin and the commercial formulation of methimazole (0.1 mL/10 mg) was applied to the inner pinna using 1 mL syringes, pre weighed to ensure accurate application of 0.1 mL. Each application of methimazole was spread across the inner ear skin with a glass rod. The donor compartment was left open to ambient laboratory conditions, to simulate conditions present for the natural absorption of the drug.

The commercial formulation of methimazole was applied as a finite dose, where a limited amount of the each formulation is applied to the skin surface, simulating conditions for the patient *in vivo*. The OECD Guideline 428 and Guidance Document 28

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(OECD, 2004a; OECD, 2004b), define finite dose absorption experiments as the application of ≤ 10 μL/cm^2 of a liquid formulation to the skin. For semisolid and solid substances, values range between 1 and 10 mg/cm^2. In this study, 10 mg of methimazole was applied to the skin, with a skin surface area of 1.13 cm^2 (8.8 mg/cm^2), criteria which would meet conditions for a finite dose under OECD guidelines.

Figure 1: Two chambered Franz type diffusion cell used in the study on percutaneous absorption of methimazole in the cat. The skin is clamped between the upper donor compartment and lower receptor compartment. The receptor compartment solution simulates the physical conditions surrounding the subcutaneous tissues. All cells were mounted in a diffusion apparatus, and placed in a water bath set to maintain the temperature of the skin in the diffusion cell at approximately 32°C. The receptor compartment solution is magnetically stirred. Samples of the receptor solution are removed via the sampling port at set time points, with equal volumes of fresh solution being replaced into the port.

Sampling time and sample collection

A 200 µL sample of reservoir solution was removed at 1, 2, 4, 6, 8, 12, 18, 24 and 30 h and was replaced by 200 µL of fresh solution into the sampling port.

The 200 µL aliquots were frozen at -20°C until subsequent analysis, conducted within 2 months of sampling.

In vitro retention

At the end of the experiment, the Franz cells were dismantled; the skin removed, rinsed thoroughly with tap water and patted dry with gauze swabs. The skin exposed to the formulation containing methimazole was excised. Ears were dissected, removing the internal pinna from the cartilage and the external pinna. These three sections were separately macerated placed in pre-weighed vials and then re-weighed. All vials were labelled and frozen at -20°C until required for further analysis, which occurred within 2 months of sampling.

Chemicals

A commercially available methimazole in a lipophilic formulation was supplied by Bayer NZ Ltd, Auckland New Zealand (Hyper-T™ Earspot, Batch number NZ05784, date of manufacture: Dec 2012, date of expiration: Dec 2013, date of study: 6 June 2013).

Bovine serum albumin was purchased from Trace Biosciences (Auckland, New Zealand), PBS from MP Biomedicals (Sydney, Australia).

Analytical methods

The methods for high performance liquid chromatography (HPLC) analysis of methimazole from serum have been previously described (Hill et al., 2011) (**Chapter 4**). Samples were analysed for methimazole concentration by a Waters HPLC (600 Model controller with 717 plus auto sampler and a 2998 Model photodiode array

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detector). For each run, 10 µL was injected at a flow rate of 0.6 mL/min onto the column (Phenomenex Luna C18, 150 x 4.6 mm, 5 µm, with a guard column) at 30°C.The detection wavelength was 252 nm, run time 15 minutes. The data was processed and integrated with Waters software (Empower™ 2). Methimazole standards were run before all samples were analysed. Limit of detection (LOD) was 20 ng/mL; limit of quantification (LOQ) was 70 ng/mL, the precision was1%. Frozen samples were thawed to room temperature and 90 µL was taken and mixed with 10 µL protein extraction solution.

The samples were vortexed and centrifuged at 14000*g* for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of 100 µL was taken for analysis.

For validation of the procedure, aliquots of (0.1 mL) of the receiver medium (PBS and BSA) were spiked with standard solutions of the commercial transdermal methimazole.

Skin Extraction

Frozen macerated skin samples were thawed to room temperature and 1 mL of water was added. The sample was left for 24 h at 4°C for methimazole desorption.

The sample liquid was taken (90 µL) and mixed with 10 µL protein extraction solution. The protein extraction solution was made of 0.1 g/mL of 5-sulfosalicylic acid in 60% water and 40% acetonitrile.

The samples were vortexed and centrifuged at 14000*g* for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of the supernatant (100 µL) was collected and analysed by HPLC as described above.

Extraction efficiency validation

The extraction efficiency (drug recovery) of methimazole from the ear skin sections was calculated by spiking blank skin samples with known amounts of methimazole. Ten skin samples where spiked with methimazole in the lipophilic vehicle at concentrations of 0.01 mg, 0.1 mg, 0.5 mg, 1 mg and 5 mg. Samples were left at room temperature (25°C) for 24 h while being gently roller mixed to permit absorption of methimazole. The methimazole was then extracted as described above and a percentage recovery determined which was then used to adjust methimazole concentrations extracted from the skin at the conclusion of the study.

Data analysis

A linear regression model was created to determine the differences between the left and right ear, and differences between the ear regions (inner ear skin, cartilage and outer ear skin). A Student t-test was used to compare the mean total concentration in the right and left ear, and to compare the thickness of the ears. Scatter plots and correlations were used to look at the relationship between methimazole concentration and the thickness of each ear region. Analyses were conducted in Stata version 12 (Statacorp LP, College Station, Texas) and $p < 0.05$ was considered significant.

One cat had an ear tattoo that penetrated the cartilage (Figure 2) and it was decided that this cat should be excluded from the analysis of the right ear due to higher methimazole concentrations measured in this cat compared with the other cats.
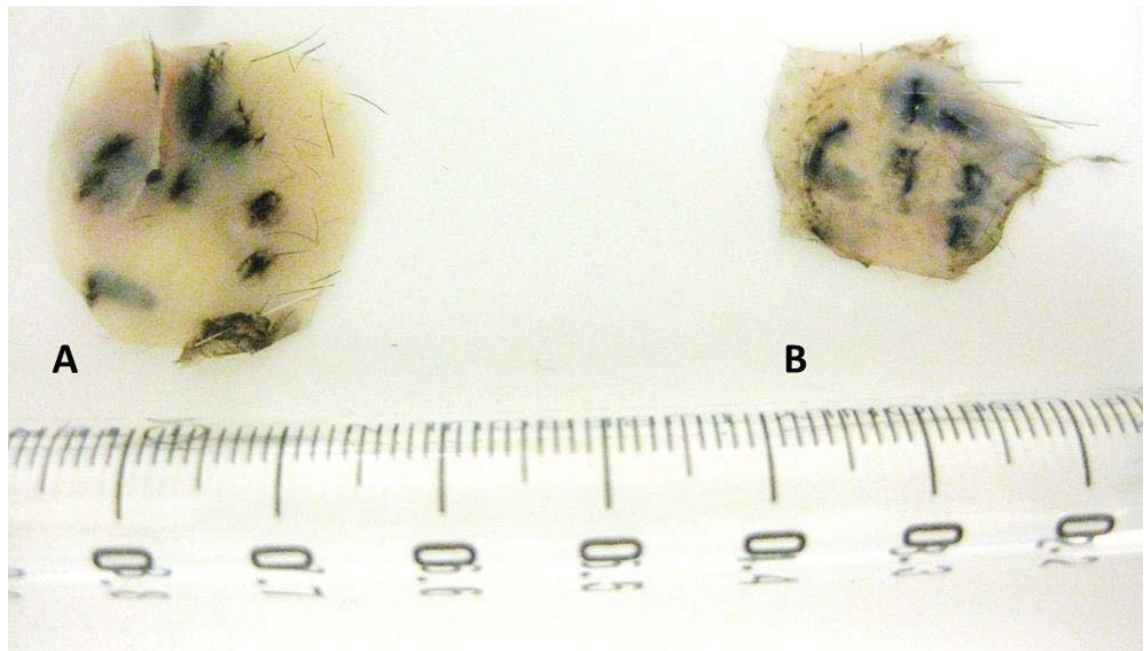
Figure 2: In an *in vitro* study showing that methimazole can cross from the inner to outer pinna of cats, one cat had a neuter tattoo that penetrated the cartilage. After application of 10 mg of methimazole to the inner pinna and the whole ear placed in an *in vitro* Franz cell for 30 h, the right ear of this cat had 3.3 mg of methimazole in the cartilage, the highest of all the cats in the study. A = Cartilage, B = inner ear.

RESULTS

A significant difference was found in the concentrations of methimazole between the left and right ear (p<0.001) (Figure 3); therefore, data from each side were considered separately. Mean ± SD methimazole concentrations for the three regions of the right and left ears, respectively, were as follows: inner ear (1.25 ± 0.53 mg/g, 0.39 ± 0.26 mg/g), cartilage (1.36 ± 0.47 mg/g, 0.33 ± 0.20 mg/g), and outer ear (1.0 ± 0.32 mg/g, 0.33 ± 0.14 mg/g). Minimal methimazole concentrations were detected in the receptor fluid (right ear 0.04 ± 0.07 mg/g, left ear 0.02 ± 0.03mg/g). The mean total amount of absorbed methimazole after application of 10 mg was 3.65 ± 1.27 mg/g for the right ear and 1.08 ± 0.27 mg/g for the left ear.

The right ear of Cat 6 (Figure 2) was excluded from the analysis, due to the extremely high concentrations of methimazole in the ear tissue - inner ear (2.8 mg/g), cartilage (3.3 mg/g), outer ear (2.1 mg/g) and total (8.3 mg/g) compared to the left ear tissue - inner ear (0.6 mg/g), cartilage (0.5 mg/g), outer ear (0.4 mg/g) and total (1.5 mg/g).

After adjusting for the effect of ear (left or right), no significant difference was found in methimazole concentration between the different regions of the ear (inner ear, cartilage, outer ear) (p=0.47) (Figure 3).

Histology of the ear tissue showed minimal evidence of significant freezing artefact such as cell disruption or tissue vacuolation. The mean thickness of the inner ear, cartilage and outer ear is shown in Table 1.

There was no difference in the mean thickness of the right ear compared with the left ear (p=0.39), the inner ear skin thickness (p=0.37), the cartilage thickness (p=0.46), or the outer ear skin thickness (p=0.48). No correlation was found with measured methimazole concentrations and the thickness of each region of the ear.

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Figure 3: The mean amount (mg/g) of methimazole recovered from six cat whole ear samples (11 ears) after application of 10 mg of methimazole in a lipophilic vehicle to the inner pinna and the whole ear placed in an *in vitro* Franz cell for 30 h, stratified by site (the right ear of one cat was excluded due to abnormally high concentrations of methimazole). The y-axis shows the amount of methimazole (mg/g), the standard deviation is shown with bars. After adjusting for the effect of ear (left or right), no significant difference was found in methimazole concentration between the different sites of the ear (inner ear, cartilage, outer ear) ($p=0.47$). A difference was found between the total methimazole concentration between the left and right ear *$p<0.001$.

Table 1: The mean thickness (µm) and standard deviation (SD) of the inner and outer ear epidermis, stratum corneum, dermis and number of epidermal cell layers from the left and right ear of six cats determined from paraffin histology sections. The mean and standard deviation (SD) of three replicates is provided. There was no difference in the mean thickness of the right ear compared with the left ear (p=0.39), the inner ear thickness (p=0.37), the cartilage thickness (p=0.46), or the outer ear thickness (p=0.48).

	Mean (*µ*m)	SD	Mean (*µ*m)	SD	Mean (*µ*m)	SD
	Left & Right		Left		Right	
Inner epidermis	28.01	6.97	24.98	6.37	31.03	6.65
Inner stratum corneum	12.91	7.63	13.83	9.69	12.00	5.67
Cell layers	3.08	0.51	3.11	0.66	3.06	0.39
Inner dermis	462.96	173.95	478.71	230.98	447.22	112.35
Cartilage	193.39	48.62	194.83	51.95	191.95	49.96
Outer dermis	568.06	170.15	561.94	194.85	574.19	160.12
Outer epidermis	25.32	4.94	27.36	4.63	23.28	4.72
Outer stratum cornem	9.58	6.35	12.42	7.62	6.73	3.34
Cell layers	2.89	0.38	3.11	0.27	2.67	0.37

DISCUSSION

The use of transdermal methimazole to treat cats with hyperthyroidism has increased over the past 10–15 years (Boothe, 2006; Trepanier, 2007; Hill et al., 2011; Boretti et al., 2013). The use of transdermal methimazole has the potential to increase the exposure of the owner of the cat who treats it with the topical methimazole preparation, and despite this risk, research into environmental and handler exposure after the use of transdermal methimazole in cats has not been published. The outcome from the current study has shown that *in vitro*, small amounts of transdermal methimazole in a lipophilic vehicle can penetrate from the inner ear, across the cartilage to the outer portion of the ear and may therefore pose a risk to people handling cats undergoing transdermal methimazole treatment.

It is not surprising that some methimazole was absorbed into the cartilage and outer ear skin in the *in vitro* conditions. The sink conditions in the reservoir solution (i.e. low concentrations of drug in the receptor fluid), would allow for passive drug diffusion (Mills et al., 2004; Mills et al., 2005; Mills et al., 2006). Over time, methimazole concentrations remained low in the receptor fluid, maintaining sink conditions, therefore the results from our study can potentially overestimate *in vivo* absorption. However, blood circulation in the skin and cartilage of the ear, such as would occur *in vivo*, would also be likely to remove drug from these areas, rather than allow drug to concentrate as occurred in the *in vitro* conditions of our study.

A technique that may be useful to confirm if methimazole is present in the outer ear stratum corneum *in vivo* is tape stripping, where corneocytes are collected on adhesive tape, extracted and then analysed for drug content (Myer & Maibach, 2013). Tape stripping is frequently used to determine drug concentrations in outer epidermal layers in human transdermal drug research and, importantly, stripping would be analogous to

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stroking or scratching the outer ear of cats. The potential for inadvertent contamination to humans owning cats treated with transdermal methimazole warrants further research as our study has exposed a potential risk.

According to manufacturers, methimazole is a suspected teratogen (Dechra, 2010). However, controversy exists, and previous published data is insufficient to draw a definitive conclusion as to the teratogenic potential of methimazole and carbimazole, with no prospective controlled studies supporting the teratogenicity of methimazole (Diav-Citrin & Ornoy, 2002). A recent study determined that there was an association between exposure to methimazole/carbimazole in the first trimester and birth defects such as omphalocele and choanal atresia (Clementi et al., 2010). The link between exposure to methimazole/carbimazole in the first trimester and these birth malformations definitely suggests that these malformations could be part of a specific, even if rare, embryopathy (Clementi et al., 2010). Follow up recommendations for the therapy of hyperthyroidism in women who are pregnant have suggested that methimazole/carbimazole be avoided in the first trimester of pregnancy, however the drug is safe for use by women in the second and third trimester of pregnancy (Cassina et al., 2012). Considering these recent recommendations (Cassina et al., 2012), the risk to pregnant women of rubbing a cat's ear that has been treated with transdermal methimazole is likely to be small, and should be further reduced by washing hands after handling the cat. However until further research is performed and considering the results of the current trial, recommendations should be made that women of child bearing age or pregnant women in the first trimester of pregnancy should wear gloves at all times when handling a cat that has been treated with transdermal methimazole.

In the current study, there was substantial inter-individual variability. High intra- and inter-individual variability is expected in *in vitro* transdermal trials (Finnin et al., 2012).

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In the current study, each cat received the same dose to the right and left ear and the left and right ears were compared to determine inter-individual differences. A difference in methimazole absorption was found between the left and right ears (Figure 3) and there was also considerable variability between subjects for the methimazole concentrations remaining in the ear (Figure 3). The differences in methimazole concentrations between the left and right ears were accounted for in the statistical model. The individual variability in methimazole absorption could be important in the clinical conditions, where variability in absorption between the left or the right ear, may alter thyroxine concentrations, and affect the resolution of clinical signs of hyperthyroidism.

Histological findings could not account for the variability in methimazole absorption in the current trial. There was no difference in the thickness of the ear regions between the left and the right ear and the thickness of the ear regions did not correlate with methimazole concentrations absorbed and measured in the different ear regions. Considerable variability was found between subjects for thickness of all the skin regions including the inner stratum corneum (Table 1). The major area of skin that provides resistance to drug penetration through the skin is the stratum corneum (Mills & Cross, 2006; Benson & Watkinson, 2012; Jepps et al., 2013), however, at least in this *in vitro* study, the thickness of stratum corneum was not related to drug penetration. In the current study, blood flow was not a factor so could also not account for the differences in methimazole concentrations absorbed in the regions of skin. Experimental variation may explain the difference in methimazole concentrations between the left and the right side as there was no obvious biological reason. Other skin factors that may affect the penetration of transdermal drugs, include the number and density of collagen fibres in the dermis or skin appendages in the epidermis (hair and sweat follicles), and further research in this area is recommended.

Another variation found in our study was the increased concentrations of methimazole in the right ear regions of the cat with a right ear tattoo which indicated it had been neutered (Figure 2). This tattoo penetrated the cartilage of the ear, and the methimazole concentrations in the cartilage and outer ear were significantly higher than those measured in the other cats. This cat was excluded from the overall analysis of the right ear tissue for this reason. However, in clinical conditions, the increased absorption of methimazole after the application of transdermal methimazole to the ear of cats with a tattoo could be significant. Ear tattooing of cats is common in many countries to indicate whether a cat is neutered, and sometimes for identification (Coalition, 2007). Cats with ear tattoos may have increased systemic absorption of drugs applied to the ear, and this requires further research.

There were limitations to this study. Only two replicates were possible per cat in the trial (only one 1.13cm^2 skin disc per ear), while the number of skin replicates recommended in permeation studies is 12, as high intra- and inter-subject variability is expected (Finnin et al., 2012). A further limitation of the current study included the use of frozen, not fresh skin. Freezing canine skin has been shown to increase the penetration of hydrocortisone by a constant rate over time, however no similar studies have been undertaken in the cat (Ahlstrom et al., 2007). However, while repeating the study with freshly harvested ears may be of interest, the ear skin in the current study was frozen for only 3 days and this short delay permitted consistent experimental conditions.

Total absorption of methimazole and subsequent recovery after application of 10 mg to the inner pinna was low (mean 3.65 mg/g right ear or 36% and 1.08 mg/g left ear or 10%), much lower than the expected 90–110% for finite dose experiments (OECD, 2004a,b). Low recovery rates in this experiment are likely due to the fact that at the

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completion of the experiment, the residual amount of methimazole on the inner pinna was washed off but a methimazole concentration was not measured to account for total drug disposition. Future experiments using finite doses of drug would ensure that all equipment and skin disc washings are included in the final absorption calculations.

CONCLUSION

This study has shown that *in vitro*, methimazole in a lipophilic vehicle will penetrate from the inner pinna to the cartilage and to the skin of the outer pinna. Care should therefore be taken when handling ears (both inner pinna and outer pinna) of cats that are treated with methimazole in a transdermal vehicle, as drug residues may be present in all layers of the skin. Women of child bearing age or pregnant women in the first trimester of pregnancy should wear gloves at all time when handling a cat treated with transdermal methimazole. Further research is required in this area to determine safety recommendations for all transdermal methimazole agents and other transdermal drugs applied to the pinna of cats.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

Kate Hill and J. Paul Chambers receive some royalties for the sales of Hyper-T™ Earspot in New Zealand.

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Chapter 8

General discussion and conclusions

PREFACE

Chapters 2–7 of this thesis present the results of a series of studies designed to test a new formulation of carbimazole and subsequently a new formulation of methimazole for transdermal application to the pinnae of cats for the therapy of hyperthyroidism. As each of these chapters has been previously published, the results are discussed within the chapter in which they are presented. Within this general discussion in **Chapter 8** the *Principal Findings* of the studies are summarised, *Discussion* of important aspects of the results is further developed, the overall *Limitations and Strengths* of this work are presented, general *Conclusions* are drawn, and *Future Directions* are suggested. This thesis includes publications that were written to stand alone. There will be some repetition of points from the discussion within individual chapters however, where possible, every effort has been made to avoid unnecessary repetition.

PRINCIPAL FINDINGS

The series of studies conducted for this thesis, evolved from the initial question, of whether PLO gel was the most suitable vehicle for carbimazole. The pilot study in **Chapter 2** established that carbimazole in a lipophilic vehicle applied to the inner pinna could be absorbed in healthy cats to a similar or greater extent than carbimazole tablets *per os*. In this study, a new, lipophilic vehicle for carbimazole was developed and HPLC methods to extract methimazole from the serum of cats were established. At the conclusion of this pilot study, a New Zealand drug company (Bomac NZ Ltd) was interested in the novel formulation and a preliminary patent was obtained. However stability testing of carbimazole in the lipophilic vehicle established that carbimazole was metabolised to methimazole within the vehicle. Therefore a second pharmacokinetic trial, outlined in **Chapter 3**, was performed in healthy cats; this time with methimazole in the lipophilic vehicle which was applied to the inner pinna. Again, concentrations of methimazole were detected in the serum of the healthy cats after the application of 5 and 10 mg of methimazole in the lipophilic vehicle applied to the inner pinna. The bioavailability of 10 mg methimazole in the lipophilic vehicle was half that of the 5 mg carbimazole oral dose. The major conclusions from this pharmacokinetic trial were that methimazole in a novel lipophilic vehicle applied to the inner pinna is absorbed to act systemically and that the pharmacokinetic profile supports the administration of 10 mg of methimazole in a novel lipophilic formulation once daily.

The results of **Chapter 4**, the clinical trial in hyperthyroid cats**,** confirmed our hypothesis that a novel lipophilic formulation of transdermal methimazole applied to the pinnae once a day, was as safe and as efficacious as oral carbimazole therapy for the therapy of cats with hyperthyroidism. The hyperthyroid cats in this clinical trial showed improvement in their clinical signs from hyperthyroidism (weight gain and reduced

blood pressure), the serum TT4 concentration was suppressed and the methimazole applied to the ear was well tolerated by the cats. In addition, owner compliance was higher with once-daily medication applied to the skin compared to twice-daily medication administered *per os*. This trial was the basis of the registration of the commercial product in New Zealand.

At the conclusion of these three studies using client owned and research cats, and as illustrated in **Chapter 1**, it was obvious that there was a dearth of knowledge in regards to whether drugs applied to the skin in cats are actually absorbed via the percutaneous route, or are perhaps systemically absorbed through ingestion via grooming. Therefore a series of *in vitro* studies were undertaken.

Chapter 5 was the first of the three *in vitro* studies and compared the percutaneous absorption pharmacokinetics of methimazole in two different vehicles, the novel lipophilic vehicle and PLO gel, using a finite dose applied to the inner ear of cat skin placed into the Franz cell model. This study confirmed that methimazole in both vehicles was absorbed across the pinnal skin. This was an important outcome, since grooming or licking has been shown to contribute to plasma drug concentrations of topically applied formulations in cats and other species. The second outcome from the study was that methimazole was absorbed more completely across the pinnal skin when administered in the lipophilic vehicle compared to administration in the PLO gel, thus adding further support that the lipophilic vehicle may be more suitable to methimazole than PLO gel.

Chapter 6 tested whether methimazole in the lipophilic vehicle could be absorbed from the skin in various skin regions of the body and whether the pinna was the most suitable site to apply topical formulations in the cat. Methimazole in the lipophilic formulation

was absorbed more completely when applied to the ear skin of cats compared to skin from the groin, neck and thorax regions, however substantial variation was seen between cats.

Finally in **Chapter 7,** a study to test the hypothesis *"Methimazole in a novel lipophilic vehicle formulation, applied to the internal pinna (non-haired region) will cross to the external (haired) pinna of the ear in an in vitro Franz type cell model."* was undertaken. The results from this study confirmed that methimazole in the lipophilic vehicle can pass from the inner pinna, through the cartilage to the skin of the outer pinna. Again, individual variation was observed within the ear skin used in the experiment, and surprisingly a difference between methimazole absorption was seen between the left and right ears, which further illustrated the individual animal variation.

In addition, in **Appendix B**, although not hypothesis driven, we have provided more information as to the measurements of inner pinnal thickness in cats. These measurements of the inner pinnal thickness may provide a foundation for further studies in regards to transdermal penetration of drugs across the pinna in cats.

DISCUSSION

The research presented in this thesis, provides the most comprehensive research to date on a drug applied to the inner pinna of cats for percutaneous absorption. The drug carbimazole/methimazole was chosen for this research as hyperthyroidism is the most common endocrinopathy in cats (Mooney, 2010), and the application of methimazole in PLO gel to the inner pinna has become an accepted alternative medical therapy for the treatment of cats with hyperthyroidism (Boretti et al., 2013).

Medical therapy is one of four options to treat hyperthyroidism in cats for which the "gold standard" therapy is still considered radioactive iodine (Peterson, 2014). Nevertheless, medical therapy remains a popular option, with a recent survey of 603 general practitioners in the United Kingdom, showing that oral medical therapy was the preferred option to treat hyperthyroidism in 65.7% of cats (Higgs et al., 2014). The nutritional option (Hills Y/D) was released during the Higgs et al. (2014) survey and transdermal therapy was not assessed as there is no licenced product for therapy of cats in the United Kingdom. The research presented in this thesis, provides veterinarians with more information when considering methimazole applied to the inner pinna, as a therapy for cats with hyperthyroidism. The application of drugs to the inner pinna has become popular in feline medicine, as cats can be difficult to consistently medicate orally (Scherk Nixon, 1996; Glerum et al., 2001; Riviere & Papich, 2001; Ciribassi et al., 2003; DeFrancesco, 2003; Hoffmann et al., 2003; Willis-Goulet et al., 2003; Mealey et al., 2004; Sartor et al., 2004; Bennett et al., 2005; Boothe, 2006; Buijtels et al., 2006; Lecuyer et al., 2006; Taboada, 2006; Helms, 2007; Plotnick, 2007; MacGregor et al., 2008). The percutaneous application of methimazole has become an popular alternative to oral therapy over the past ten years (Boothe, 2006; Trepanier, 2007; Hill et al., 2011; Boretti et al., 2014), and research into the safety and efficacy of this formulations was

required. Research into the application of methimazole to the inner pinna, should not be interpreted as recognition that percutaneous therapy is being recommended as the "gold standard" for the therapy of hyperthyroidism in cats. However, there is obviously a demand for safe and convenient options to supplement the current treatment options for hyperthyroidism, as medical therapy is still the most popular choice to treat cats with hyperthyroidism in the United Kingdom (Higgs et al., 2014).

An important outcome from **Chapters 2** and **3** was that carbimazole and subsequently methimazole was percutaneously absorbed for systemic action in healthy cats. In **Chapter 3**, the mean bioavailability of 10 mg methimazole in the lipophilic vehicle (48% [minimum 43, maximum 55]) applied to the inner pinna relative to twice daily oral carbimazole could be calculated in all five cats (**Table 1, Chapter 3**), whereas a previous study of methimazole in PLO gel, could determine bioavailability in only two of six cats because of low serum concentrations (Hoffman et al., 2002). Bioavailability was able to be calculated in all five cats in our study because: 1. the lipophilic vehicle has increased the absorption of the methimazole; and 2. a higher dose of methimazole (10 mg) was used in our study compared with 5 mg of methimazole in PLO gel in the study by Hoffman et al (2002).

In **Chapter 4**, the systemic absorption of methimazole in the lipophilic vehicle was further proven by the detection of serum methimazole concentrations after administration (**Figure 2, Chapter 4**), and the reducing serum TT4 concentrations (**Figure 1, Chapter 4**). However, the methimazole concentrations correlated poorly with concurrent serum TT4 concentrations in both groups (**Figure 3, Chapter 4**). It was discussed in **Chapter 4**, that methimazole concentrates in the thyroid, therefore serum methimazole concentrations are unlikely to be correlated with serum TT4 concentrations and that the endpoint of successful treatment for these types of anti-

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thyroid drugs, and indeed all hyperthyroid treatments, can be determined by their biological effect, i.e. the decrease in TT4 concentration and resolution of clinical signs rather than the serum concentration of methimazole alone. Irrespective of the drug concentrations in serum after transdermal administration, there is uncertainty following the topical application of methimazole as to whether the drug penetrates solely through the skin (or the ear in this case) or if there is some degree of oral ingestion after grooming of the application site by the cat (Hoffmann et al., 2003). Grooming or licking has been shown to contribute to plasma drug concentrations of topically applied formulations in cattle (Toutain et al., 2012) and other species (Sallovitz et al., 2012). For this reason, the study in **Chapter 5** was conducted, to prove that methimazole in the lipophilic vehicle did indeed cross the skin, and this will be discussed later.

Chapter 4 showed results of dose adjustments with transdermal methimazole, which is the first time this has been reported. Dose adjustments with oral methimazole are not uncommon, with the dose adjusted to maintain a serum TT4 concentration within the reference range. In 80 cats treated with oral methimazole over 30 to 100 days, the final median maintenance dose of methimazole was 10 mg per day (range 5 to 20 mg per day) (Peterson et al., 1988). In **Chapter 4** eight cats in the transdermal group had dose modifications (**Table 2, Chapter 4**). Five cats had a dose reduction and three cats had a dose increase. Thirteen cats in the oral carbimazole had dose modifications, with nine requiring a decrease in the dose and four requiring an increase. The median dose of transdermal methimazole at 12 weeks was 10 mg (range 3 to 15 mg). In the long term study by Boretti et al 2014, the requirements for methimazole in PLO gel changed over time. The median dose at 24 to 36 months was 7 mg (range 2.5 to 10 mg) was higher than the starting dose of 5 mg (range 2.5 to 5) ($p<0.05$). Dose adjustments occurred in 50 cats, with an increase in dose in 26 cats and a decrease in 24 cats. Further

adjustments had to be made after the initial changes, with the dose of eleven of the 26 cats that first had an increased dose requiring a decreased dose, and 13 of the 24 cats that first had a dose decrease requiring the dose to be increased at later rechecks. Dose changes with both oral and transdermal methimazole is expected over time, as thyroid nodules can increase in size after long term therapy with methimazole (Peterson, 2012). However other changes can occur that may affect transdermal absorption, such as skin blood flow changes, alterations in the permeability of the skin after chronic application of the formulation and possibly even concurrent skin diseases. The effect of these cutaneous changes is currently unknown and requires further investigation.

In **Chapter 5,** we proved that methimazole in PLO gel and the lipophilic vehicle can be absorbed through the skin of the inner pinna (**Figure 1, Chapter 5**) using an *in vitro* Franz cell model. Important to the main hypothesis of this thesis, the rate and extent of the penetration through the ear was formulation dependent, with the methimazole in the lipophilic vehicle absorbed to a greater degree than the methimazole in the PLO gel. The findings from **Chapter 5**, gave further support to our overall hypothesis, that the PLO gel may not be the most suitable vehicle for carbimazole/methimazole.

As discussed in **Chapter 4**, the skin site chosen for the application of methimazole, might change the rate of drug delivery, a factor that had previoulsy been investigated in dogs, but not cats (Mills et al., 2004; Mills et al., 2005; Mills et al., 2006). It was for this reason that the study in **Chapter 6** was conducted. The conclusion from **Chapter 6**, that the ear is the preferred area for this lipophilic formulation of methimazole, compared to the neck, groin and thorax (**Figure 1, Chapter 6**), is important practical knowledge for veterinarians who use this formulation. The inner ear also has the lowest chance of the cat (or owner) rubbing the ointment from the ear before the drug is absorbed. Further

work is required for all drugs in gel formulations to see if the inner ear is the best site for all drugs and all formulations.

Our work in **Chapter 7**, was important, as the use of transdermal methimazole has the potential to increase the exposure of the owner of the cat who treats it with the topical methimazole preparation. Research into environmental and handler exposure after the use of transdermal methimazole in cats had not been published prior to our study. The main result from **Chapter 7** has showed that *in vitro,* small amounts of transdermal methimazole in a lipophilic vehicle can penetrate from the inner ear, across the cartilage to the outer portion of the ear and may therefore pose a risk to people handling cats undergoing transdermal methimazole treatment (**Figure 3, Chapter 7**). *In vivo* blood flow to the ear is likely to reduce the concentration of methimazole to the outer pinna, however the exact concentrations of methimazole in the outer pinna of live cats treated with transdermal methimazole is currently unknown. Until further research is conducted to clarify the relative risk of small amounts of methimazole residue in the skin to owners, care should be taken when handling ears (both inner pinna and outer pinna) of cats that are treated with methimazole in a transdermal vehicle, as drug residues may be present in all layers of the skin.

Overall, the results of this thesis provide a body of work that supports the systemic availability of methimazole in a lipophilic vehicle, both using *in vitro* and *in vivo* studies in cats, and that the formulation is safe and effective for clinical use in hyperthyroid cats. The studies prove that the choice of vehicle is important for the percutaneous absorption of methimazole in cats. Although the most comprehensive body of work to date on a particular drug for the percutaneous application to the inner pinna, the thesis also reveals that there is research still to be performed, and further studies and researched is discussed below in *Future Directions*.

LIMITATIONS AND STRENGTHS

The studies presented in this thesis, represent the most extensive studies to date of a drug applied to the inner pinna of cats. The results from these studies prove that methimazole in a lipophilic vehicle is absorbed across the skin of cats, and that the systemic effects of methimazole, are not entirely from ingestion via self-grooming of the drug. We found that methimazole in the lipophilic vehicle was an efficacious therapy for cats with hyperthyroidism. Importantly, these studies highlight the fact that each cat is an individual as we saw substantial inter-individual variation in the absorption of methimazole across the skin, particularly in the *in vitro* Franz type cell studies. However, there were limitations to all of the studies.

One of the primary limitations of the thesis, was sample size. For the two pharmacokinetic studies (**Chapter 2** and **3**), the number of cats used was small, but comparable to similar pharmacokinetic studies in cats (Frenais et al., 2008; Wells et al., 2008). Although an increase in cost is associated with increasing the number of cats, it would be recommended that future pharmacokinetic studies should increase the sample size. Most veterinary pharmacokinetic studies (Kim et al., 2014; Pelligand et al., 2014; Quimby et al., 2014) still tend to use six to eight cats. Sample size for pharmacokinetic studies can be calculated using power calculators or specific calculations for pharmacokinetic studies, which take into consideration parameters such as inter-subject variability (Kang et al., 2005).

Chapters 2 and **3** also tested whether the transdermal formulations were equivalent to the oral formulations. To accurately determine sample size for bioequivalent studies, an appropriate sample size calculation should be performed, which takes into consideration various parameters such as the mean expected difference between the test and reference formulations and the anticipated intra-subject variance for the parameters stated in the

standard (EMA, 2010). Type I error rate should be set at 5% and power for the study should be calculated. The minimum number of subjects is usually 12, however often more are required (EMA, 2010). The use of excessive number of animals, does come at an ethical cost, therefore it is important to calculate numbers of animals required for each study, and the aim of the study (i.e. pharmacokinetic or bioequivalence or both). Future studies that compare transdermal formulations with oral formulations, should aim for at least 12 cats, although all factors, such as intra subject variability and ethical consent from the institution, will need to be taken into consideration.

A lack of skin replicates was also discussed as a limitation of **Chapters 5-7**. For the studies in **Chapters 5-7**, ethics approval was only obtained for 18 cats, the results from our research would support the use of larger numbers of cats. Increasing the number of skin samples could decrease the large intra-subject variability. However, multiple skin replicates from each cat ear were not possible as only one 1.13 cm^2 skin disc could be obtained from each ear. Therefore a larger number of cats would be required for future studies, unless a smaller Franz cell was used, requiring a smaller skin disc size. Thus, increasing cat numbers using our current model, could further increase the intra-subject variability.

Another limitation of **Chapters 2** and **3** was that an intravenous dose of methimazole or carbimazole was not administered, thus the absolute bioavailability could not be calculated. Importantly, in the **Chapters 2** and **3**, no blood samples were taken between 24 and 148 hours therefore the pharmacokinetics during this period remain unknown. For medications applied to the skin, bioequivalence should be assessed after single and multiple doses, as suggested by the European Medicine Agency (Anonymous, 2010), and any future studies should have a full pharmacokinetic profile after multiple doses applied to the skin.

A limitation of the studies in **Chapters 2-4** was that the thyroxine (TT4) concentration range was the range established by the laboratory, and was not validated for these studies. A minimum of 120 samples is recommended to establish reference intervals by non-parametric methods with 90% confidence intervals and in future studies, a validated thyroxine concentration should be used (Friedrichs et al., 2012).

Another limitation of the clinical trial in **Chapter 4**, is that the cats were not randomly assigned to each treatment group, they were alternately assigned, which is considered a deterministic method of randomisation. Alternate assignment can still introduce bias as the person recruiting trial participants knows the next treatment and may be influenced in the recruitment process (Chalmers, 1999). Appropriate randomisation is important so that a deliberate element of chance into the assignment of treatments is introduced and also produces treatment groups in which the distributions of prognostic factors (known and unknown) are similar (EMA, 2012). The clinicians in our study, were also not blinded to the therapy, however this would be difficult considering the difference between oral tablets and ointment for application to the skin. The group sizes were small for a phase II study (20 in each group), however again this sample size is comparable to other veterinary phase II clinical trials (Skorupski et al., 2011; Saba et al., 2012; Paterson et al., 2014). A placebo group was also not included, although inclusion of a placebo or control group would have difficulties gaining ethics approval by the Massey University Animal Ethics Committee for this particular study.

Several limitations were identified and discussed in **Chapters 5-7**. Future work with cat skin frozen for longer than five months would require further studies to ensure that there was no increase in permeability of the drug (Ahlstrom et al., 2007).

In **Chapters 5-7**, the unabsorbed dose of methimazole was not accounted for, which includes the methimazole that remained on the skin, as well as any drug bound to the Franz cells and experimental equipment. Future studies should include washes from the skin and also the equipment, to account for the unabsorbed dose of drug (OECDa, 2004; OECDb, 2004).

Another limitation determined from the skin histology, was the variations in stratum corneum thickness (**Chapters 5-7** and **Appendix B**). The variation could have been due to tissue handling during the process of formalin fixation or from individual variation between cats. As discussed in **Appendix B,** The formalin-paraffin processing may be unsuitable for the determination of stratum corneum thickness due to the disruption of the horny layer during preservation process (Therkildsen et al., 1998). More recent studies measuring stratum corneum thickness in humans have used reflective confocal microscopy or cryoprecipitation preservation of tissue rather than formalin fixation (Sandby-Moller et al., 2003; Robertson & Rees, 2010). Future studies to measure the stratum corneum thickness in cats using one of these newer methods is recommended.

CONCLUSIONS

The results of the research presented in this thesis provided evidence that:

1. Methimazole in a novel lipophilic formulation for application to the inner pinna of cats can be absorbed into the systemic circulation in healthy cats.

2. Methimazole in a novel lipophilic formulation for application to the inner pinna of client owned hyperthyroid cats is efficacious and safe.

3. A finite dose of methimazole in a novel lipophilic formulation and methimazole in PLO gel are absorbed across the pinnal skin in an *in vitro* Franz cell model.

4. Methimazole is absorbed more completely across the pinnal skin when administered in the novel lipophilic vehicle compared to methimaozle in PLO gel, using a finite dose in an *in vitro* Franz cell model.

5. A finite dose of methimazole in the novel lipophilic formulation was absorbed more completely when applied to the ear skin of cats compared to skin from the groin, neck and thorax regions, in an *in vitro* Franz cell model.

6. Methimazole in the novel lipophilic formulation can pass from the inner pinna, through the cartilage to the skin of the outer pinna in an *in vitro* Franz type cell model.

FUTURE DIRECTIONS

There are many questions that arise from the results presented in this thesis. These

questions include:

1. What are the pharmacokinetics, bioavailability and bioequivalance of

   methimazole in a lipophilic vehicle applied to the inner pinna of cats after

   multiple doses?

This question may be more academic in nature, as the drug will still be dosed to affect

(ie the declining serum TT4 concentration) in the hyperthyroid cat. However this

question was not answered by the study in **Chapter 3,** as only one sample (at 148

hours) was taken after multiple doses.

2. What is the long term (>12 week) efficacy of a large number of hyperthyroid

   cats treated with methimazole in a lipophilic vehicle applied to the inner pinna?

This question is anecdotally being answered by the continued use of the commercial

product in New Zealand, however a long term study such as the one conducted by

Boretti et al 2014, with methimazole in PLO gel, has not been conducted (Boretti et al.,

2014). Ideally, a prospective, 12 month study with monitoring of cats every three

months would be performed.

3. Does the dose requirement of hyperthyroid cats treated with methimazole in a

   lipophilic vehicle applied to the inner pinna change significantly over time?

As shown in the long term study by Boretti et al 2014, the requirements for

methimazole in PLO gel changed over the 24 to 36 month follow up period. It is

unknown whether dose changes are required over a long term period with the

methimazole in the lipophilic vehicle and whether the chronic application of this

formulation to the ear skin may affect absorption and therefore alter dose requirements over time.

4. Is there a gender difference in absorption of drugs applied percutaneously to the ear of cats?

A gender difference of absorption was not noticed in the clinical trial, however some gender differences were noticed in the *in vitro* trials. Larger numbers of cats will be required both *in vitro* and *in vivo* to answer this question.

5. Does freezing cat skin alter percutaneous drug absorption *in vitro*?

As discussed in the section on limitations, and in the various relevant chapters, it is currently unknown if freezing cat skin, and the length of time the skin is frozen, will increase drug absorption *in vitro*.

6. Do skin appendages (such as hair follicles) of cats have a role in the percutaneous absorption of drugs?

The inner pinna of the cat is relatively hairless, however from the results from **Chapter 6** suggest that this site is still the best site for the absorption of methimazole in the lipophilic vehicle. Skin appendages can increase absorption of other drugs absorbed from the skin, therefore further investigation into this area would be useful to maximise absorption of drugs via the skin in cats.

7. Are there any long term effects of the application of methimazole to the skin?

With the chronic application of methimazole to the skin, could there be any long term effects such as malignant transformation or the induction of skin disease.

8. Do other skin diseases (such as pyoderma, ear mites etc) change the absorption of methimazole applied to the skin?

9. What amount of methimazole remains on the skin of cats after the percutaneous application to the inner pinna and for how long *in vivo*?

10. Is there a safety issue for people after drugs, applied to the skin of the inner ear crossing to the outer pinna?

11. Is the inner pinna the best site of application for all drugs in gel formulations?

The questions above could apply to any drug applied to the skin of the inner pinna of cats, and individual drugs will need to be investigated to determine their absorption characteristics and safety. The answers to these questions will help to clarify whether transdermal drug therapy in cats is truly safe and efficacious for both the cat, the owner of the cat and the environment.

The use of drugs applied to the inner pinna of cats has increased substantially over the past ten years, and this method of therapy has proved popular with the owners of some cats that are difficult to medicate. Further work is required in all aspects of transdermal drug therapy in cats to advance the science in this field. It is hoped that the results from this thesis has somewhat contributed to the advancement of knowledge in this area. The work from this thesis has shown some promising results. Providing that this promise is born out in the long term large scale use of methimazole in a lipophilic vehicle, the formulation has the potential to significantly increase the quality of life of a large and growing number of hyperthyroid cats and their owners.

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Appendix A

General methods for the *in vitro* skin penetration experiments

INTRODUCTION

This appendix outlines the materials and methods used for the *in vitro* skin penetration studies performed in **Chapters 5,6** and **7**. There is some repetition describing these methods in **Chapters 5**,**6** and **7**; as the chapters were published as manuscripts submitted for publication in a journal and were prepared in accordance with the "advice to authors" for the journal in which they were published. However, the methods described in this appendix includes a full description as some elementswere excluded in the published paper. The appendix also includes some further exploration of the data, by combining some of the results of **Chapters 5** and **6.** The *in vitro* studies described in this appendix, were undertaken at the University of Queensland, School of Veterinary Science, Gatton Campus, Queensland Australia.

MATERIALS AND METHODS

Animals

Eighteen domestic short haired cats (6 male and 12 females) were euthanized at a local pound by an intravenous injection of sodium pentobarbital, and the skin and pinnae harvested within 4 h after death. The sex of the cat was determined by physical examination, 3 male cats were entire, 3 male cats were neutered, 3 female cats had an ear tattoo indicating they were neutered, and 9 female cats did not have an ear tattoo which classified them as entire females.

This research protocol was approved by the Animal Ethics Committee of the University of Queensland (approval number SVS/494/12).

Skin collection

Eighteen pairs of feline ears were harvested within 4 h after death using metzenbaum scissors, removing the entire ear and all cartilage at the base of the ear. Ears were labelled with the date, cat identification number, and left or right, placed into a plastic bag and frozen at -20°C until required (range 3 days to five months). Hair was trimmed from the inner ear using curved metzenbaum scissors. Ear skin was allowed to defrost for 1 h at room temperature (25°C) and for preparation for studies in **Chapters 5** and **6**; transected with gentle blunt dissection to separate the ventral (inner surface) from the dorsal aspect of the ear. For the experiment in **Chapter 7**, whole ears were used, therefore no transection was performed.

Subcutaneous and cartilage tissue was removed from the ears for studies detailed in **Chapters 5** and **6**. The ear skin was rinsed with tap water to remove any adherent blood and dried gently with a gauze swab. One circular section measuring 2 cm^2 was cut from each ear using a round biopsy punch and mounted onto the diffusion cells (Figure 1).

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The remainder of the skin surrounding the hole was trimmed for histology. For the experiment in **Chapter 7**, whole ears were used, therefore no transection was performed.
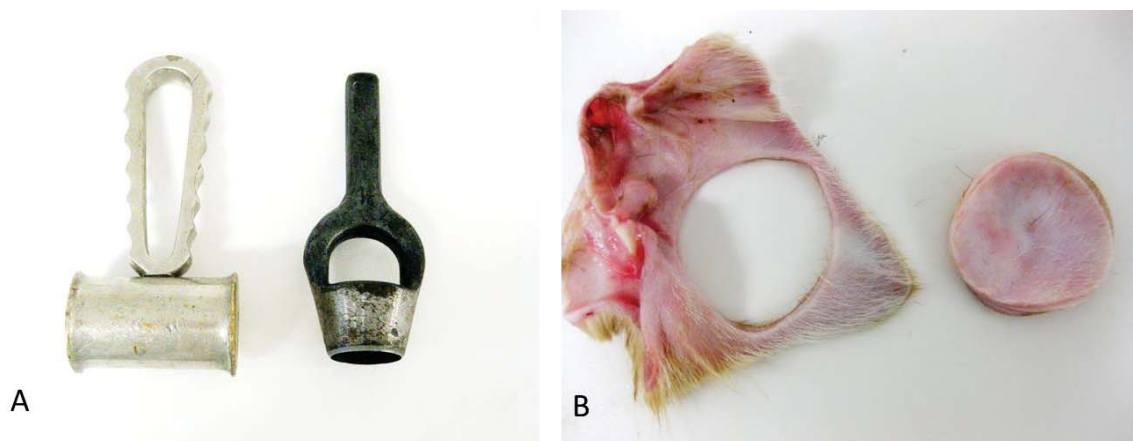
Figure 1: The round biopsy punch and mallet (A) used to cut a circular section measuring 2 cm² from each ear, as shown for the whole ear studies in **Chapter 7** (B).

Full thickness skin from the groin (defined as caudal to last nipple), thorax (defined as the dorsoventral line intersecting the caudal angle of the scapula; dorsal border: transverse processes of the thoracic vertebrae; caudo-ventral border: an arc joining the dorsal border from the last rib, to the cranial border at the level of the costo-chondral junction), neck (defined as caudal to the wing of the atlas and cranial to the scapula, with dorsoventral borders drawing a line between atlas and scapular) (Ahlstrom et al., 2007). To harvest the skin, the hair was removed using clippers, (clippers: Oster®, model 078005-140-070; blades: Oster® CryotechTM, size 40) taking care not to damage the skin surface. The skin was dissected using metzenbaum scissors and all the subcutaneous fat was removed. Skin was labelled with the date, cat identification number, and region of origin, placed into a plastic bag and frozen at -20°C until required (within five months). Skin was allowed to defrost for 1 h at room temperature

(25°C) and was then visually inspected for any deficits (such as holes in the skin) (Ahlstrom et al., 2007). For each region of skin (neck, groin and thorax) a skin biopsy punch was used to cut two x 2 cm^2 circles of tissue. The skin samples were washed with tap water and dried with gauze swabs and then mounted onto diffusion cells. The remainder of the skin was trimmed for histology to determine epidermal and dermal thickness.

The number of skin replicates usually recommended in permeation studies is 12 (Finnin et al., 2012). This replication is required to the high intra- and inter-subject variability. If groups are being compared, matched skin samples should be used (Finnin et al., 2012). The number of replicates in the **Chapters 5-7** varied between experiments. For **Chapter 5**, only one skin disc (i.e. one replicate) could be obtained from each cats' ear, for each formulation due to the size of the ear and the dimensions of the sample of skin required for the Franz cell. In **Chapter 5**, to limit inter-subject variability, skin from the left and right ear of each cat (i.e. matched skin samples) was used to apply the different vehicle samples, therefore each cat served as its own control. For **Chapter 6**, one skin disc from the left and right ear of each cat, provided two replicates, and two replicates were used from the 3 other skin regions. In **Chapter 7**, one skin disc from the left and right ear, again provided two ear replicates. In all chapters, histology of the ear skin was performed to determine any physical causes of intra-subject variability.

Effect of freezing on skin samples

A decision was made to freeze the skin samples since the skin and ears were collected opportunistically as cats were euthanased by a local pound and this collection method was acceptable to the Animal Ethics Committee at the University of Queensland. Samples had to be collected when animals were available. Freezing skin has a small but predictable effect to increase the permeability of some drugs in both the dog and the

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sheep (Ahlstrom et al., 2007; Bayldon, 2012). The time the skin and ear skin was frozen for the current studies (**Chapters 5-7**) would have been unlikely to have affected drug permeability. Other authors have concluded that as long as skin is not overly hydrated when frozen, freezing is unlikely to significantly alter drug permeation characteristics compared with non-frozen skin (Finnin et al., 2012).

The effect of freezing cat skin on *in vitro* methimazole permeability was not specifically examined for the studies in **Chapters 5-7**, however histology was performed to determine if any freezing artefact could be seen and whether there were any differences between cats or any skin pathology that could influence drug permeability. From the previous studies outlined above, a decision was made that short term(< 5 months) freezing of cat skin should have minimal effects on drug permeability.

Chemicals

Chemicals to make the Methimazole in PLO were purchased from Sigma Aldrich (St Louis MO USA). Methimazole in a lipophilic formulation was supplied by Bayer NZ Ltd, Auckland, New Zealand (Hyper-T™ Earspot Batch number NZ05784, Date of manufacture Dec 2012, Expiration Dec 2013, date of studies June 2013).

Bovine serum albumin (Premium Grade; 303050) was purchased from Trace Biosciences NZ Ltd, Hamilton, NZ through Thermo Trace Ltd, Noble Park, VIC, Australia. Phosphate Buffered Saline tablets, (Sodium chloride 8.0 g/L, Potassium chloride 0.2 g/L, Disodium hydrogen phosphate 1.15 g/L, Potassium dihydrogen phosphate 0.2 g/L) from MP Biomedicals Inc. (Ohio, USA). Acetonitrile HPLC grade was from Merck, Germany, and 5-sulfosalicylic acid HPLC grade, from Sigma Aldrich, St Louis, MO USA.

Selection of receptor fluid (all diffusion cell studies)

As we saw from **Chapter 1**, the receptor solution must have adequate solubility for the compound being studied. In accordance with the Guidance document for the conduct of skin absorption studies (Organisation for Economic Co-operation and Development (OECD), 2011), solubility of the test substance in the receptor solution used in the Franz-type diffusion cells should not be a rate-determining step in skin absorption. Methimazole and carbimazole are lipophilic substances and the OECD guidelines state :

"Lipophilic substances are poorly soluble in most receptor fluids, and partitioning will be inhibited. In vivo, lipophilic compounds are readily taken up by blood once it enters the cutaneous capillaries. The receptor fluid used in vitro should serve the same role as blood does in vivo. However,unlike in vivo conditions, the receptor fluid volume may be more limited, particularly in static diffusion cells. The effect of this can be minimised by use of frequent sampling (and subsequent replacement with new receptor fluid, as should be done in studies of this type)."

For the reasons stated above, 4% bovine serum albumin was added to phosphate-buffered isotonic saline (sodium chloride 8.0 g/L, potassium chloride 0.2 g/L, disodium hydrogen phosphate 1.15 g/L, potassium dihydrogen phosphate 0.2 g/L) (PBS) (MP Biomedicals, Sydney Australia), to make the receptor fluid for the methimazole diffusion studies (Sartorelli et al., 2000).

In vitro skin penetration studies

The experimental procedure for the *in vitro* skin penetration studies were similar to procedures previously described, with skin and ear skin mounted onto Franz-type diffusion cells with the stratum corneum uppermost (**Chapter 1**, Figure 1.5.1 (Mills et al., 2004; Mills et al., 2005; Mills et al., 2006; Ahlstrom et al., 2007; Ahlstrom et al., 2009; Ahlstrom et al., 2010; Ahlstrom et al., 2011; Ahlstrom et al., 2013)). The surface area of skin exposed to drug in the diffusion cells was 1.13 cm^2. The dermal chamber was filled with 3.5 ml of a reservoir solution suitable for a lipophilic drug, consisting of phosphate-buffered isotonic saline (PBS) (sodium chloride 8.0 g/L, potassium chloride 0.2 g/L, disodium hydrogen phosphate 1.15 g/L, potassium dihydrogen phosphate 0.2 g/L) (MP Biomedicals) with 4% bovine serum albumin (BSA) (Sartorelli et al., 2000), pH 7.4±0.1and the donor compartment left open to ambient laboratory conditions. All Franz cells were mounted in a diffusion apparatus with the dermal bathing solution being stirred by a magnetic plate stirrer with the temperature of the skin in the diffusion cell approximately 32°C (**Chapter 1**, Figure 1.5.2 water bath). After mounting, the skin was hydrated for an hour (h) with sodium phosphate buffer (0.1 M) and the system was allowed to equilibrate. The buffer solution was aspirated from the surface of the skin and the formulation/s of methimazole applied (the exact dose and type of preparation is described in each chapter). Each dose was spread across the skin with a glass rod. The donor compartment was left open to ambient laboratory conditions, to simulate conditions for the *in vivo* absorption of the drug.

Sampling time and sample collection

A 200 µL sample of reservoir solution was removed at 1, 2, 4, 6, 8,1 2, 18, 24 and 30 h (**Chapters 5** and **7**) and 36 h in **Chapter 6**, and 200 µL of fresh PBS and 4% BSA solution was replaced into the sampling port.

The aliquots (200 µL) were frozen at -20 °C until subsequent analysis, within 2 months of sampling.

In vitro retention

At the end of the experiment, the cells were dismantled, the skin samples removed, rinsed thoroughly with tap water and patted dry with gauze swabs. The unabsorbed drug dose should have been removed from the skin during this process. The skin exposed to the applied formulation was excised and macerated with scissors. Macerated samples were placed in pre-weighed vials, and accurately weighed. Samples were labelled and frozen at -20 °C until required for further analysis.

Analysis of samples

Instrument conditions

Samples were analysed for methimazole concentration by a Waters HPLC (600 Model controller with 717 plus autosampler and a 2998 Model photodiode array detector). For each run, 10 µL was injected at a flow rate of 0.6 mL/minute onto the column (Phenomenex Luna C18, 150 x 4.6 mm, 5 µm, with a guard column) at 30°C. The detection wavelength was 252 nm, run time 15 minutes. The data was processed and integrated with Waters software (Empower™ 2). Methimazole standards were run before all samples were analysed. Frozen samples were thawed to room temperature and 90 µL was removed and mixed with 10 µL protein extraction solution. The protein

extraction solution was made of 0.1 g/mL of 5-sulfosalicylic acid in 60% water and 40% Acetonitrile.

The samples were vortexed and centrifuged at 14000 x g for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of 100 µL was taken for analysis.

Skin Extraction

Frozen samples were thawed to room temperature and 1 mL of water added. The samples were left for 24 h at 4°C. A 90 µL aliquot was taken from each vial and mixed with 10 µL of protein extraction solution. The protein extraction solution was made of 0.1 g mL-1 of 5-sulfosalicylic acid in a 60% water and 40% Acetonitrile.

All samples were vortexed and centrifuged at 14000 x g for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of the supernatant (100 µL) was collected and analysed by the HPLC as described above.

Recovery efficiency validation

To calculate the recovery efficiency of methimazole from the skin, the amount of methimazole in the lipophilic vehicle and methimazole in PLO gel was calculated from skin samples spiked with known amounts of methimazole in each vehicle. Ten skin samples where spiked with methimazole in PLO or lipophilic vehicle at concentrations of 0.01 mg, 0.1 mg, 0.5 mg, 1 mg and 5 mg. Samples were left at room temperature (25°C) for 24 h to allow complete absorption of methimazole. The methimazole was then extracted from the skin using the methods described above and an average percentage of methimazole recovery from the skin was determined for each vehicle (85% for the lipophilic vehicle and 37% for the PLO vehicle, Table 1). To calculate the true amount of methimazole that was retained in the skin of experimental samples at the

conclusion of the studies the amount of methimazole extracted was divided by the average extraction efficiency of the formulation applied to the skin.

Table 1: Ten skin samples were spiked with methimazole in PLO or lipophilic vehicle at concentrations of 0.01 mg, 0.1 mg, 0.5 mg, 1 mg and 5 mg. The methimazole was then extracted from the skin using HPLC analysis and an average percentage of methimazole recovery from the skin was determined for each vehicle.

Methimazole concentration added to skin	Percentage extracted from skin (%)	
	Lipophilic vehicle	PLO vehicle
0.01 mg	60	55
0.1 mg	86	37
0.5 mg	94	37.2
1 mg	93	29
5 mg	94	28
Average percentage extracted	85.4	37.2

Data Analysis

The concentration of methimazole in the receptor fluid at each collection period was measured. To calculate the true methimazole concentration at each time point, a correction factor was applied which accounted for the dilution factor caused by 200 µL being removed from the 3.5 mL chamber and replaced with 200 µL of PBS and BSA:

(measured HPLC concentration) Concentration at time 1 = x µg/mL

Diluted concentration at time 1 after 200 µL is replaced $= x \times \frac{3.3}{3.5}$

(measured HPLC concentration) Concentration at time 2 = y µg/mL

True (corrected for dilution at time1) concentration at time 2 $= y + \left[x - x \times \frac{3.3}{3.5}\right]$

The flux (J) was calculated using the formula J = Q/(At) (Franz et al., 2009)

Where Q is the total quantity of compound traversing the membrane in time t, and A is the area of exposed membrane in cm^2. For these experiments, A was constant at 1.13 cm^2

The cumulative amount of methimazole that had permeated the skin to the receptor fluid was calculated for each replicate at each sampling time point.

Cumulative methimazole data was explored for trends (such as gender differences) between **Chapters 5** and **6** for the lipophilic formulation. Linear regression models were created to test the difference between the results from the two chapters for cumulative amount of methimazole. Data is shown as both mean and standard deviation

and geometric mean and 95% confidence intervals. A value of p<0.05 was considered significant. Results are presented at the end of this appendix.

Total absorption of methimazole

Due to the limited amount of drug applied, it is essential in the finite dose experiment that a mass balance is performed. OECD (2011) guidelines state $100\% \pm 10\%$ drug recovery is required for drug registration.

The definitions for total absorption are included below.

Unabsorbed dose: represents the mass of the test substance washed from the skin surface after exposure and any present on the nonocclusive cover, including any drug dose shown to volatilise from the skin during exposure to the ambient environment. The unabsorbed dose was not accounted for in the studies described in **Chapters 5-7**.

Absorbed dose: the mass of the test substance reaching the receptor fluid or systemic circulation within a specified time.

The absorbable dose: represents the mass of the test substance present on or in the skin after washing of the skin.

To determine the overall recoveries of the *in vitro* experiment the following : unabsorbable dose from skin washings and *in vitro* cell washings, skin absorbable dose, absorbed dose in receptor fluid, should be subtracted from the applied dose. In the subsequent chapters a true mass balance could not be determined as skin surface and the Franz cell washings were not performed at the conclusion of the experiment.

Therefore in the following chapters, the mean total absorption of methimazole was calculated from the addition of the methimazole concentration in the receptor fluid (absorbed dose of drug) to the methimazole recovered from the skin samples

(absorbable dose of drug , i.e. the dose of drug remaining in the skin). Other procedures, such as tape stripping, may have been useful to estimate what proportion of absorbed drug was contained in the stratum corneum, although once in this protective layer, an active drug would generally be considered 'absorbed' (Escobar-Chavez et al., 2008). Tape stripping is also difficult to perform in *in vitro* studies over 24 h duration (OECD, 2011).

Statistical analysis and any further data analysis relevant to the chapter are detailed in the Materials and Methods section of each chapter.

RESULTS OF DATA EXPLORATION

Cumulative methimazole concentrations

The combined results of the cumulative methimazole concentrations for the methimazole in the lipophilic vehicle from **Chapters 5** and **6** are shown in Figure 2 and 3, expressing the data as both means and geometric means. There was no difference between the two studies expressing the data as mean (p=0.88) or geometric mean (p=0.81). The data divided into male (n=4) and female (n=8) cats is shown in Figure 4 and 5 expressing the data as both means and geometric means. No difference was found between the two sexes expressing the data as mean (p=0.94) or geometric mean (p=0.76).
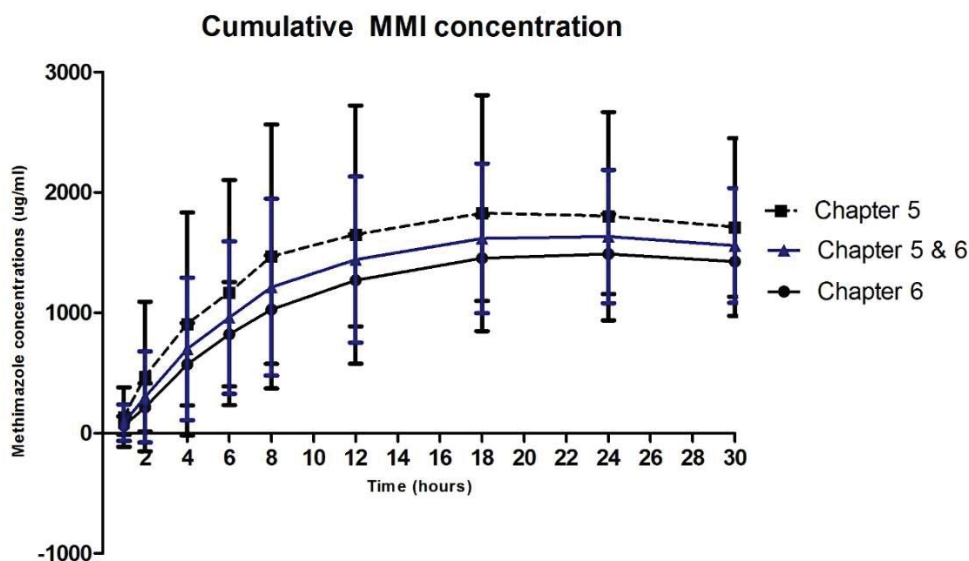
Figure 2 : Cumulative methimazole (MMI) concentration using an *in vitro* Franz cell model, after the application of 10 mg of MMI in a lipophilic vehicle to the right inner pinna skin of six cats(■) (**Chapter 5**) or six pairs of inner pinnal skin discs (●) (**Chapter 6**) and the data from both **Chapter 5** and **6** combined (▲). Data shown as mean and the error bars represent standard deviation. There was no difference between the two studies (p=0.88).

Figure 3: Cumulative methimazole (MMI) concentration using an *in vitro* Franz cell model, after the application of 10 mg of MMI in a lipophilic vehicle to the right inner pinna skin of six cats(■) (**Chapter 5**) or six pairs of inner pinnal skin discs (●) (**Chapter 6**). Data shown as geometric mean and the error bars represent 95% confidence intervals. There was no difference between the two studies (p=0.81).

Figure 4: Cumulative methimazole (MMI) concentration using an *in vitro* Franz cell model, after the application of 10 mg of MMI in a lipophilic vehicle to the inner pinna skin of four male cats (●) and eight female cats (■). No difference was found between the two sexes (p=0.94). Data shown as mean and the error bars represent standard deviation.
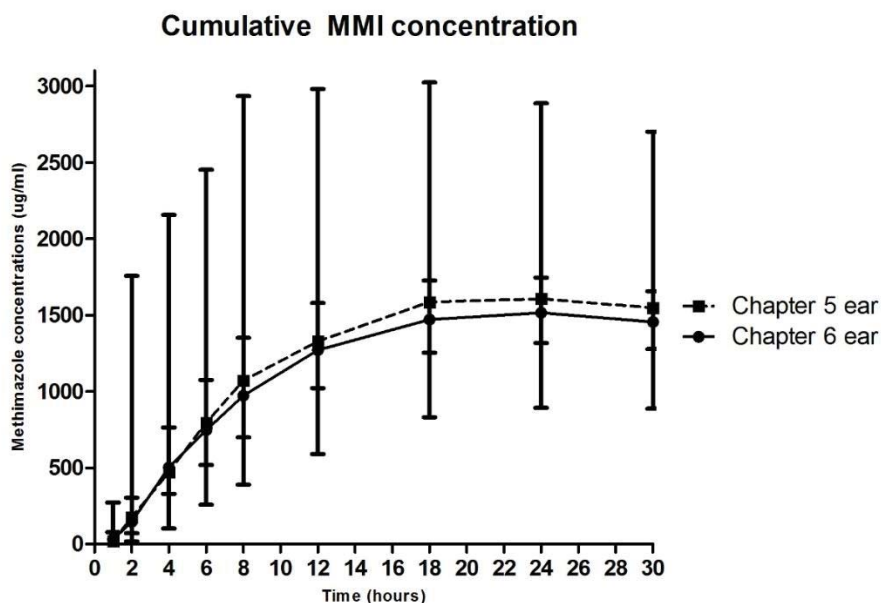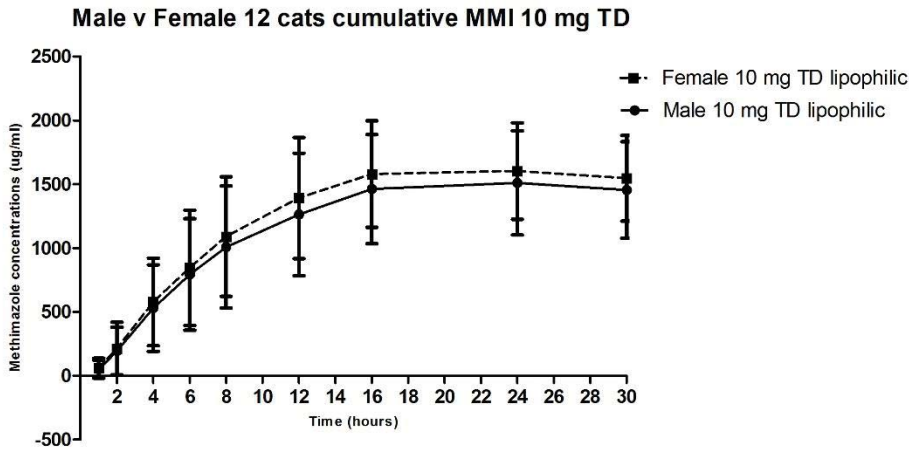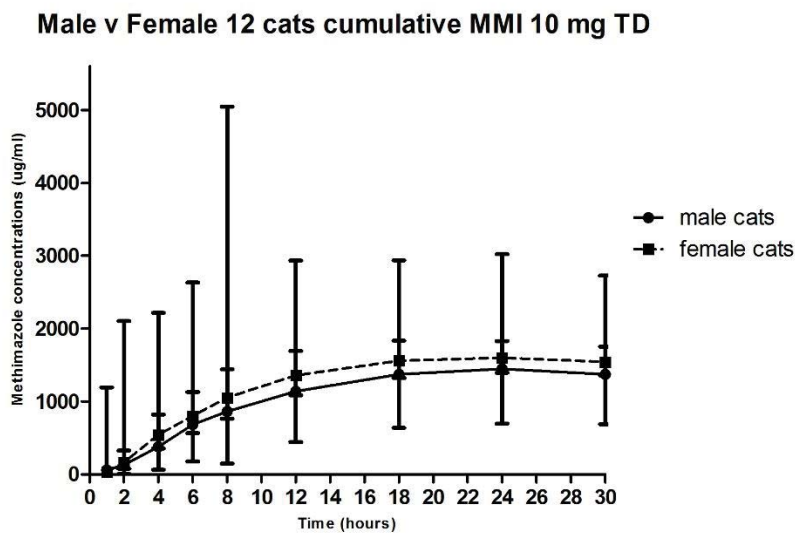

Figure 5: Cumulative methimazole (MMI) concentration using an *in vitro* Franz cell model, after the application of 10 mg of MMI in a lipophilic vehicle to the inner pinna skin of four male cats (●) and eight female cats (■). No difference was found between the two sexes (p=0.76). Data shown as geometric mean and the error bars represent 95% confidence intervals.

DISCUSSION

In **Chapter 5,** we saw considerable individual variation in the cumulative concentration of methimazole between the 6 cats (**Chapter 5**, Figure 3), therefore cumulative methimazole concentrations were explored for trends by combining results from **Chapters 5** and **6** for the methimazole in a lipophilic vehicle. Overall there was no difference between the two studies (Figure 4 and 5) and no differences were found between males and females for the cumulative methimazole concentrations (Figure 6 and 7).

In **Chapter 5**, the inner pinnal skin from the two older intact male cats was less permeable to the lipophilic formulation of methimazole compared to the younger cats, however the permeability of methimazole between the two vehicles for the intact male cats was comparable (**Chapter 5**, Figure 4). In **Chapter 5**, statistical analysis of the effect of age and neuter status, was not performed due to the small sample size (n=2) and the large confidence intervals. By combining the data from the two studies, no gender difference was found. However, the sample sizes are still small (n=4 males, n=8 females), and further research into whether age, gender and neuter status and the type of vehicle affects percutaneous drug absorption would still be recommended, as discussed further in **Chapter 5**.

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Appendix B

General methods for the histology of skin samples used in the *in vitro* skin penetration experiments

INTRODUCTION

This appendix outlines the materials and methods used for the histology of the skin samples from the *in vitro* studies performed in **Chapters 5,6** and **7**. There is some repetition of these methods in **Chapters 5,6** and **7**; as each chapters was prepared for publication as journal manuscripts in accordance with the journal's requirements. However, the methods described in this appendix includes detailed methodology as some elements of which were removed from the materials and methods in the published papers. The appendix also includes exploration of the data with all histological results from the ear samples from the 18 cats combined together. The *in vitro* studies described in this chapter, were undertaken at the University of Queensland, School of Veterinary Science, Gatton Campus, Queensland Australia.

MATERIALS AND METHODS

Animals

Eighteen domestic short haired cats (6 male and 12 females) were euthanized at a local pound by an intravenous injection of sodium pentobarbital, and the skin and pinnae harvested within 4 h after death as described in Appendix A. This protocol was approved by the Animal Ethics Committee of the University of Queensland (approval number SVS/494/12).

Skin collection

Skin was harvested as outlined in Appendix A.

Histology

Samples of the skin and ear surrounding the hole left from the biopsy punch were trimmed and fixed in 10% buffered formalin for 24-48 h, processed and embedded transverely into paraffin blocks. Haematoxylin and eosin (H&E) staining was performed on 3μm sections of tissue cut from the formalin-fixed, paraffin-embedded (FFPE) blocks for light microscopy.

Histological analysis was performed using a Nikon Eclipse N*i* microscope. Histological images were captured using NIS Elements software (Nikon Instruments Inc., Melville, NY, United States) and analysed using Image J software (Schneider et al., 2012). Exact regions and areas measured are identified in each chapter. The inner pinna was always examined, and in **Chapter 8** the cartilage and outer pinna were examined as well (Figure 1). For each sample, each region (i.e. the stratum corneum, epidermis etc) were measured (in microns (μm)) or counted at three randomly selected locations and the measurements averaged as previously described (Monteiro-Riviere et al., 1990) (Figure 2 and 3). Both left and right ears of each cat were examined.

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To determine if there were any differences in the inner pinna of the 18 cats, the various skin regions, sex of the cat, and left and right side of the ear were compared using Student t-test. A value of $p < 0.05$ was considered significant.
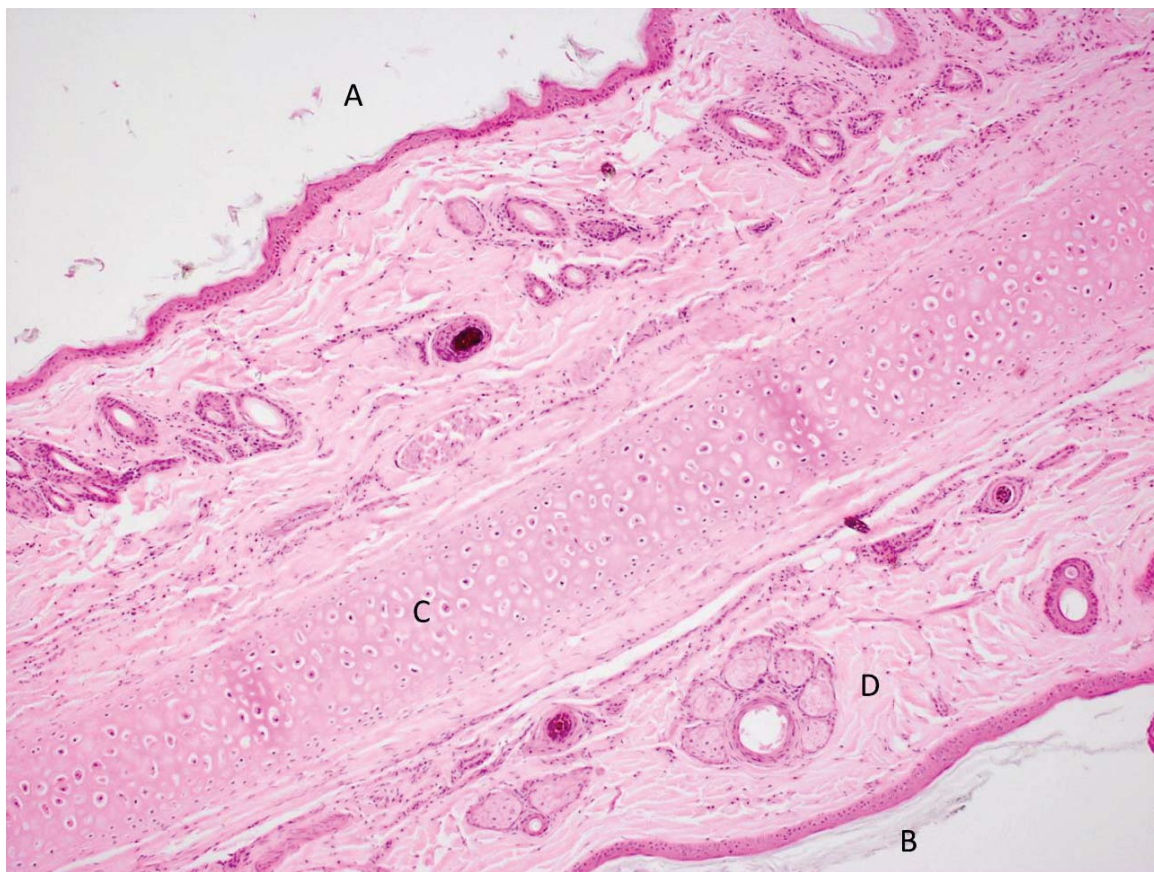
Figure 1: Haematoxylin and eosin (H&E) stain of cat pinna (40 x magnification). A = the outer pinna, B = the inner pinna, C = the cartilage of the ear and D = the dermis. To measure thickness of the epidermis, dermis, cartilage and stratum corneum, three areas were measured and then averaged.

Figure 2: Haematoxylin and eosin (H&E) stain of cat neck skin (40x). To measure each skin region,three regions were measured in microns (µm) and averaged. (Monteiro-Riviere et al., 1990). An example of three measured regions of stratum corneum (cornified, non viable epidermis) (SC 1–3) and viable epidermis (E 1–3), are shown in this figure.

Figure 3: Haematoxylin and eosin (H&E) stain of cat inner pinna skin (100x). To measure each skin region, three regions 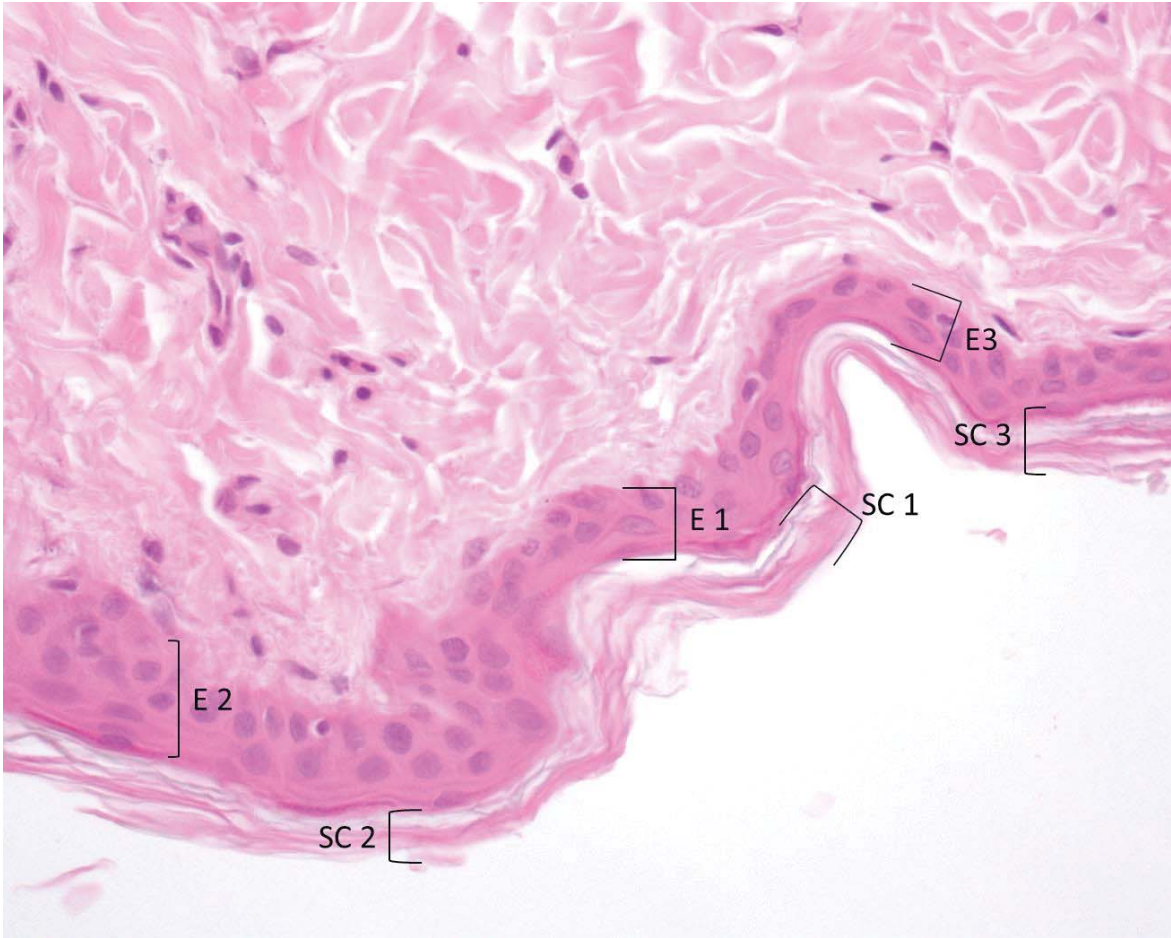were measured in microns (µm) and averaged. (Monteiro-Riviere et al., 1990). An example of three measured regions of stratum corneum (cornified, non viable epidermis) (SC 1–3) and viable epidermis (E 1–3), are shown in this figure.
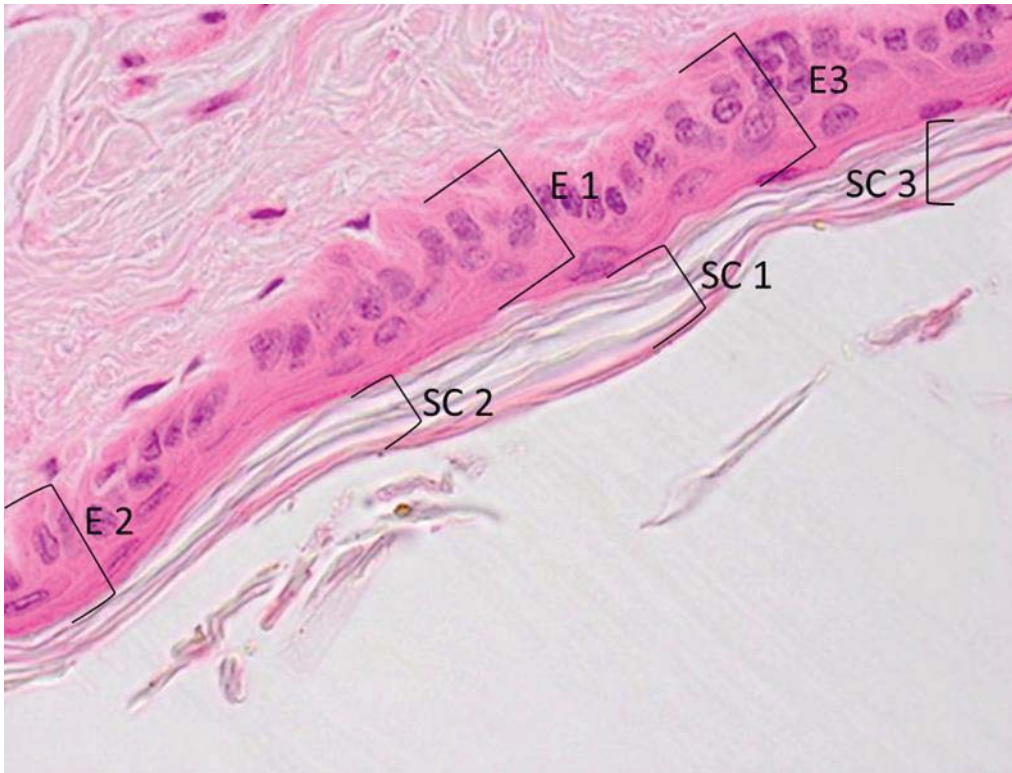
RESULTS

Individual measurements for the inner pinna of eighteen cats are shown in Table 1.
Considerable individual variation in thickness of the skin regions was found between
the cats as shown in Table 1 and Figure 4. Table 2 shows the total mean thickness (µm)
and standard deviation (SD) of the inner ear epidermis and stratum corneum and
number of epidermal cell layers from the left and right ears of the eighten cats as
determined in 3µm paraffin histology sections. In Table 2, the cats are also divided into
sex (male or female) and left and right side of the ear. No difference was seen between
the two sexes (p=0.09 for the epidermis and 0.16 for the stratum corneum) or between
the left and the right sides of the ear for each region of the epidermis (p=0.28 for the
epidermis and 0.48 for the stratum corneum).

Table 1: The mean thickness (µm) and standard deviation (SD) of the inner ear epidermis and stratum corneum and number of epidermal cell layers from the left and right ears of eighteen cats as determined in 3 µm paraffin histology sections. To measure each skin region, three regions were measured in microns (µm) and averaged. F=Female. M = Male. MC = castrated, FS = spayed. L = Left side, R = Right side.

Cat	Sex	Side	Epidermis (*µm*)		Stratum Corneum (*µm*)		Layers	
			Mean	SD	Mean	SD	Mean	SD
1	F	L	18.38	7.61	4.3	1.84	2.67	0.58
1	F	R	12.64	3.81	1.77	0.95	3.33	0.58
2	F	L	15.46	1.74	17.46	10.51	2	0
2	F	R	12.73	1.03	17.24	9.48	2	0
3	F	L	18.23	4.03	10.53	2.04	2.67	0.58
3	F	R	11.14	3.77	7.7	2.16	2.33	0.58
4	F	L	15.42	0.41	9.89	3.84	2	0
4	F	R	12.78	5.08	7.2	1.04	2.33	0.58
5	F	L	16.24	2.28	13.72	1.58	2.67	0.58
5	F	R	13.8	4.51	6.07	0.98	2.33	0.58
6	MC	L	13.67	0.89	9.73	2.88	2.33	0.58
6	MC	R	12.32	4.9	13.38	2.23	2	0
7	M	L	12.92	1.06	1.23	0.23	3.33	0.58
7	M	R	12.64	3.81	1.77	0.95	3.33	0.58
8	M	L	11	1.73	3.42	3.52	3	1
8	M	R	17.55	4.66	11.52	4.92	3.33	0.58
9	FS	L	36.87	6.61	3.18	0.77	5.67	0.58
9	FS	R	38.55	2.64	3.5	1.59	5.67	0.58
10	FS	L	22.24	0.81	2.64	1.11	3.33	0.58
10	FS	R	39.39	6.59	3.21	21.91	5	1
11	MC	L	26.3	10.66	3.71	1.09	3	1
11	MC	R	23.05	3.66	2.24	0.65	3	0
12	FS	L	27.42	3.59	3.25	1.12	3	0
12	FS	R	13.99	1.76	1.42	0.71	2	0
13	F	L	36.87	7.75	13.5	9.28	4	1
13	F	R	36.6	6.09	12.31	6.29	3.33	0.58
14	MC	L	20.08	2.37	13.48	2.66	2.67	0.58
14	MC	R	27.95	2.95	18.6	9.12	2.67	0.58
15	M	L	25.22	9.29	5.44	2.19	2.67	0.58
15	M	R	28.81	4.39	4.3	1.46	3	0
16	F	L	24.87	3.3	15.74	3.56	2.33	0.58
16	F	R	41.55	9.18	8.15	1.51	3.67	0.58
17	F	L	18.97	2.15	31	15.77	3.67	0.58
17	F	R	27.47	6.07	18.34	3.39	2.67	0.58
18	FS	L	23.9	2.7	3.81	1.05	3.33	0.58
18	FS	R	23.79	2.65	10.28	5.33	3	0

Table 2: The total mean thickness (µm) and standard deviation (SD) of the inner ear epidermis and stratum corneum and number of epidermal cell layers from the left and right ears of eighten cats as determined by 3 µm paraffin histology sections. To measure each skin region, three regions were measured in microns (µm) and averaged. F=Female. M = Male. L = Left side, R = Right side. No significant difference was found between male and female or left and right ears for any epidermal region.

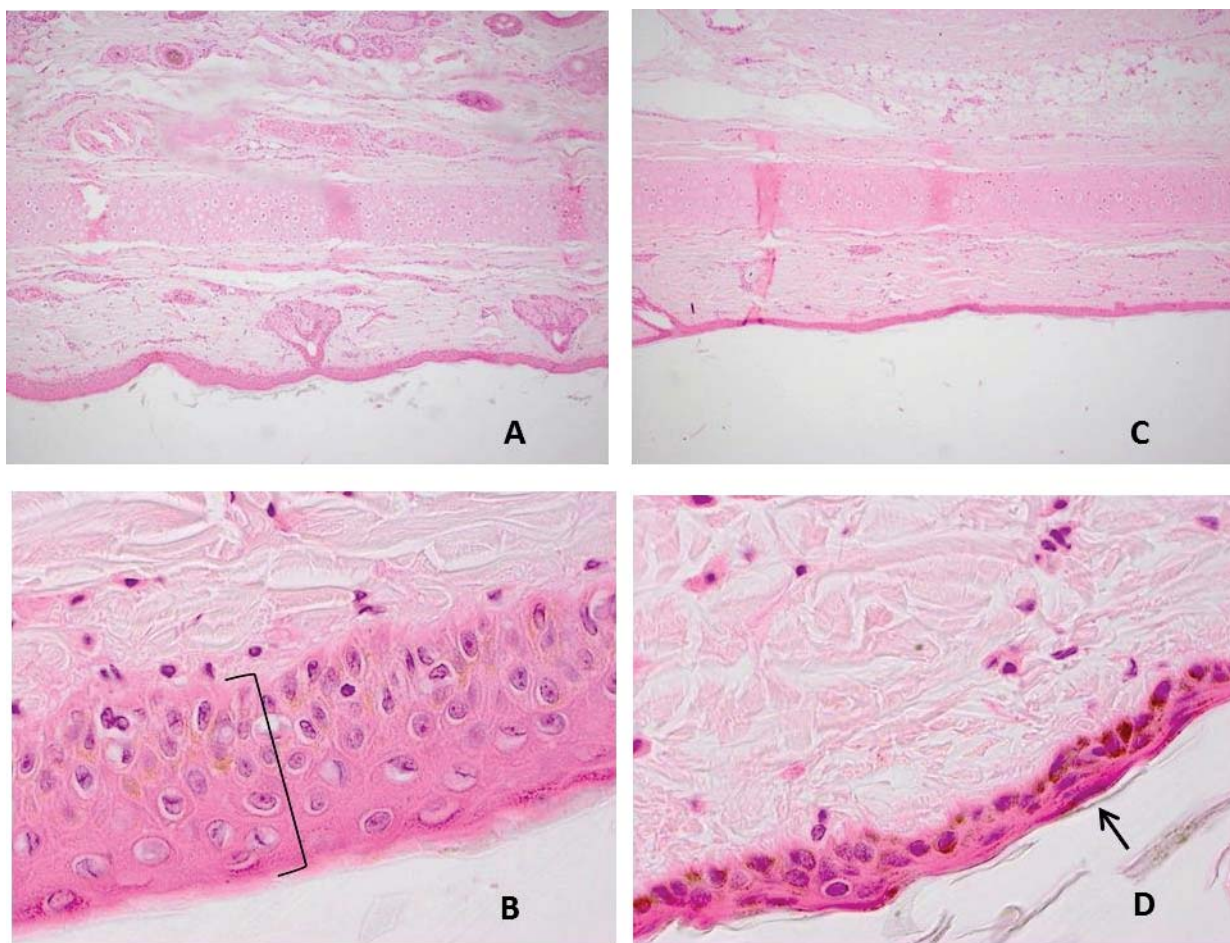|	Epidermis (μm)		Stratum Corneum (μm)		Cell Layers	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
All cats (n=18)	22.23	9.16	8.95	6.63	3.03	0.92
Male cats (n=6)	19.30	6.80	7.40	5.10	2.90	0.40
Female cats (n=12)	12.80	10.00	9.80	7.10	3.10	1.10
M v F p value	0.09		0.16		0.22	
Left Inner Ear	21.34	7.42	9.22	7.49	3.02	0.85
Right Inner Ear	23.18	10.86	8.66	5.80	3.04	1.01
L v R p value	0.28		0.40		0.47	

Figure 4: Haematoxylin and eosin (H&E) stain the inner pinnae of cats. Considerable variation was seen between 18 cats where H&E stain was performed on the inner pinnae. (A) and (B) show the inner pinna of the left ear of cat 9 (A = 100x) (B = 1000x) in the study and (C) and (D) the inner pinna of the right ear of cat 12 (C = 100x, D = 1000x) demonstrating that cat 12 had a thinner epidermis compared to cat 9 and a very thin stratum corneum (indicated by the arrow).

DISCUSSION

The goal of this study was to measure inner pinnal thickness in cats. This study will provide a foundation for further investigations where the inner pinnal thickness is required, particularly in regards to transdermal penetration of drugs across the pinna in cats.

In **Chapter 5**, we identified that the two intact male cats had a thickened dermis in one cat, and a slight increase in dermal thickness in the other (**Chapter 5**, Table 2). Similar gender differences in skin thickness have been noted in other species, including humans where males have thicker skin in some regions, compared to females, while female skin generally thins after menopause (Giacomoni et al., 2009). A question arose from **Chapter 5**, whether gender differences in histology existed, particularly for the stratum corneum. Therefore the data available from the ear histology of all 18 cats was combined. No difference was found between male and female cats by combining the data.

In **Chapter 7**, a difference between methimazole concentrations was found in the left and right ear, however no difference was found between the thickness of the left and right ear measured by histology. By combining the data of all 18 cats, we found no difference in the left and right ear thickness for the epidermis and stratum corneum.

As discussed in **Chapter 5**, in the study by Monteiro-Riviere et al (1990), the stratum corneum thickness (3.94 µm ± 0.44) epidermal thickness (14.71 µm ± 1.36) and number of cell layers (1.39 ± 1.01) were different from the current study. However, the identified differences may be due to the methods used as it is unknown whether the inner or external epidermis and stratum corneum of the ear were examined, and the gender of the experimental cats was not provided (Monteiro-Riviere et al., 1990).

An important finding from the current study was the individual variation in skin region thickness between the cats. No statistical difference was found between the thickness of the skin in males and females or between the left and right ears, however great variability was seen between the cats and sometimes between the ear of each cat, when comparing the findings in the left and right ears. One limitation of the study was that these cats were from a pound and their exact age or neuter status could not be determined. Male cats were assigned to the castrated group if no testicles were visible and if there was an ear tattoo, however it is possible but still unlikely that some cats may have been cryptorchid. Female cats were assigned to the spayed category if there was an ear tattoo indicative of neuter status in Queensland.

The differences in the thickness of the stratum corneum seen in this study may have been related to tissue handling during the process of formalin fixation. The formalin-paraffin processing may be less suitable for the determination of stratum corneum thickness due to this disruption of the horny layer during preservation (Therkildsen et al., 1998). More recent studies measuring stratum corneum thickness in humans have used reflective confocal microscopy or cryoprecipitation preservation of tissue rather than formalin fixation (Sandby-Moller et al., 2003; Robertson & Rees, 2010). Future studies to measure the stratum corneum thickness in cats using one of these newer methods is recommended.

In conclusion, by combining data from all 18 cats for histological analysis, we are confident that 1) considerable individual variation exisits between cats and sometimes within cats 2) there are no differences between the thickness of the left and right ear 3) no differences in thickness of the epidermis and stratum corneum of the ear for male and female cats.

From our experience with histological investigations of the ears of cats, we recommend further research on determination of the thickness of the different skin regions of the cat, in particular the stratum corneum. A larger number of cats should be sampled, to try and decrease the individual variation. Accurate data on the gender and neuter status of the cat will also be required. To measure the thickness of the stratum corneum accurately, formalin-paraffin preservation is unsuitable and newer methodology such as confocal microscopy or cryoprecipitation preservation would be recommended.

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Appendix C

General methods for high performance liquid chromatography (HPLC)

Introduction

For the studies in this thesis, a simple and sensitive method for the detection of methimazole by high performance liquid chromatography with diode array detection was performed. The method was modified from that of Trepanier *et al* (1991). The method was refined over the course of the whole project, and there are minor differences with the method used in the pilot study (**Chapter 2**).

MATERIALS AND METHODS

Reagents

Methimazole (minimum 98%) was obtained from Sigma Aldrich, New Zealand. The working standard solution was prepared fresh every day in mobile phase. The mobile phase consisted of ammonium acetate 0.1M (Sigma Aldrich, New Zealand) in 5% (v/v) acetonitrile (Lichrosolv, Merck, New Zealand) in Milli-Q water (Milli-Q PFplus system, Millipore, USA) adjusted to pH 4.0 with HCl and filtered through a 0.22μm filter. Methanol (Lichrosolv) was also obtained from Merck. All other chemicals were obtained from Sigma Aldrich.

Sample preparation

Blood

Whole blood samples from cats were allowed to clot for at least 30 minutes. Blood samples were stored overnight at 4°C and serum was prepared by centrifugation at 3,000 × g for 5 minutes and the supernatant collected. Serum was stored at -20°C until use. Proteins in the serum samples were removed by precipitation with methanol. To 100 μL of serum, 500 μL of ice cold methanol was added and the sample vortexed. Samples were incubated on ice for 30 minutes and then centrifuged at 10,000 x g for 5 min. The supernatant was collected and then air dried on a heating block at 55°C. The dried samples were dissolved in 100 μL of mobile phase, vortexed and sonicated briefly and centrifuged at 10,000 × g for 10 minutes. The supernatant (100 μL) was collected and loaded into the auto sampler. All samples were prepared in duplicate for HPLC.

Skin

Frozen macerated skin samples were thawed to room temperature and 1 mL of water added. The sample was left for 24 hours at 4°C for methimazole desorption.

The sample liquid was taken (90 µL) and mixed with 10 µL protein extraction solution. The protein extraction solution was made of 0.1 g/mL of 5-sulfosalicylic acid in 60% water and 40% acetonitrile.

The samples were vortexed and centrifuged at 14 000 g for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of the supernatant (100 µL) was collected and analysed by the HPLC.

Franz cell receiver fluid

Frozen samples were thawed to room temperature and 90 µL was taken and mixed with 10 µL protein extraction solution. The protein extraction solution was made of 0.1 g/mL of 5-sulfosalicylic acid in 60% water and 40% acetonitrile.

The samples were vortexed and centrifuged at 14000g for 20 minutes. An additional 100 µL of dimethyl sulfoxide was added to neutralize the acid. An aliquot of 100 µL was analysed.

For the validation of the procedure, aliquots (0.1 mL) of receiver medium (PBS and BSA) were spiked with standard solutions of transdermal methimazole in lipophilic vehicle and PLO gel.

Chromatographic conditions

Serum samples were analyzed using a Shimadzu LC20VP system, skin and Franz cell fluid by a Waters 600 Model controller with 717 plus auto sampler and a 2998 Model photodiode array detector. For each run, 10 µL was injected at a flow rate of 0.6

mL/min onto the column (Phenomenex Luna C18, 150 x 4.6 mm, 5 μm, with a guard column) at 30°C. The detection wavelength was 252 nm and the run time 15-20 minutes. Samples were analysed in triplicate.

Data analysis

Data was analysed in either LC Solutions (Shimazu) or Empower 2 (Waters) software. The standard calibration curves were also processed in the same software.

Validation

Limit of quantification

Dilutions of methimazole in mobile phase were made until no signal was detectable. The limit of detection (LOD) was set at 3 times the noise (20 ng/mL); the limit of quantification (LOQ) was set at 10 times the noise (70 ng/mL).

Intra-day and inter-day accuracy and precision

The inter-day accuracy and precision was determined by running standard in mobile phase and in the spiked plasma at four different concentrations (5, 10, 20 and 40 ng/mL) every day for 3 days. The intra-day accuracy and precision was calculated from these data. Intra-assay variation was ± 3.1% at a 95% confidence level. Inter-assay variation at a 95% confidence level ranged between ± 1.5% and 9.0% over the range of concentrations.

Linearity

The linearity of the detector response to the drug was checked by running 6 different concentrations of methimazole (1.25 - 40 ng/mL) in mobile phase and in serum. These data were analysed by linear regression in LC Solutions software (Shimadzu). The response was linear from 0.9989-0.9999 ng/mL (figure 1).
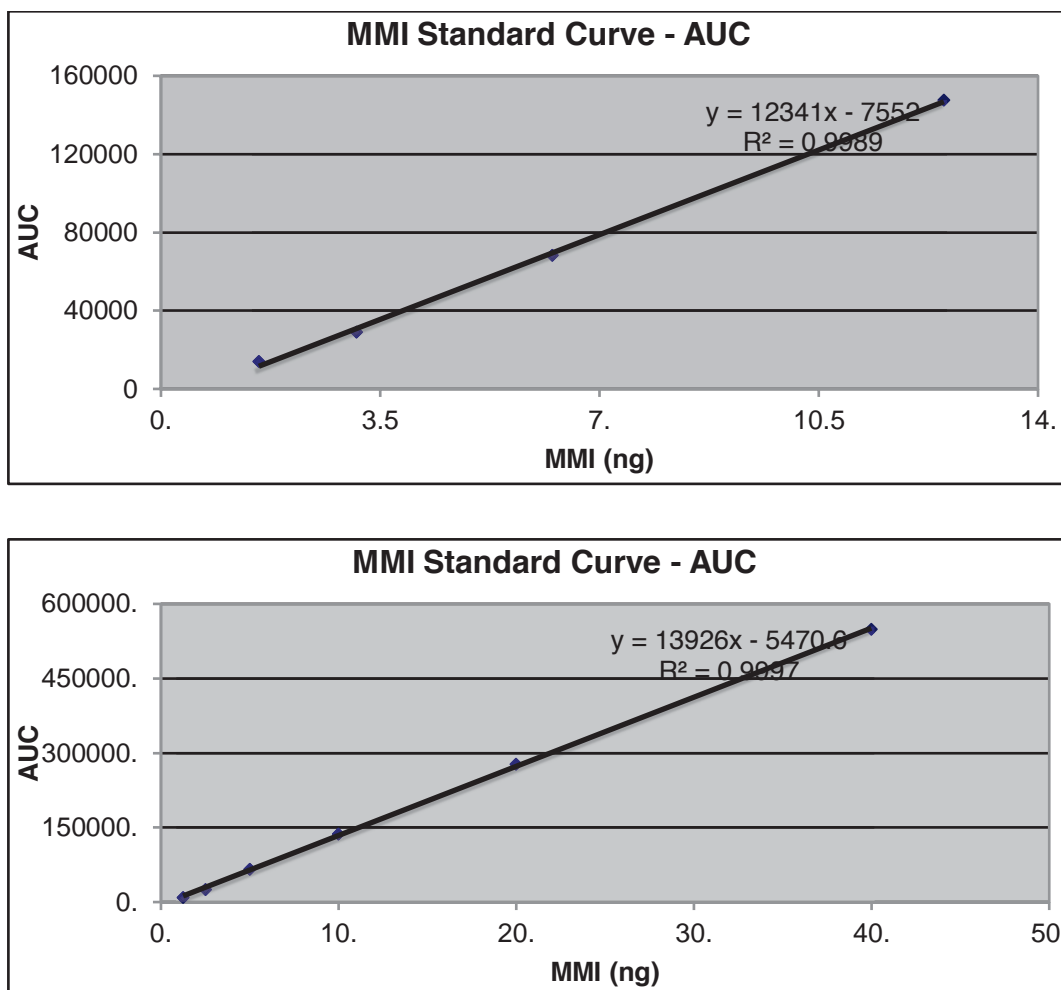
MMI Standard Curve - AUC

y = 12341x - 7552
R² = 0.9989

MMI Standard Curve - AUC

y = 13926x - 5470.6
R² = 0.9997

Figure 1: Examples of checking the linearity by running 6 different concentrations of methimazole (1.25 - 40 ng/mL) in mobile phase and in serum from cats.

Recovery

The recovery of methimazole after extraction from serum was determined by comparing the area of the peaks for 1.25, 2.5, 5, 10, 20, 4 ng/mL methimazole in mobile phase with the areas for the same concentrations spiked in the various matrices. To calculate the recovery efficiency of methimazole from the skin, the amount of methimazole in the lipophilic vehicle and methimazole in PLO gel was calculated from skin samples spiked with known amounts of methimazole in each vehicle. Ten skin samples where spiked with methimazole in PLO or lipophilic vehicle at concentrations of 0.01 mg, 0.1 mg, 0.5 mg, 1 mg and 5 mg and described in **Appendix A.**

Specificity

Blank plasma from 3 different cats was analysed to check that no other peaks eluted at the same time as methimazole. No interfering peaks were detected (figure 2).
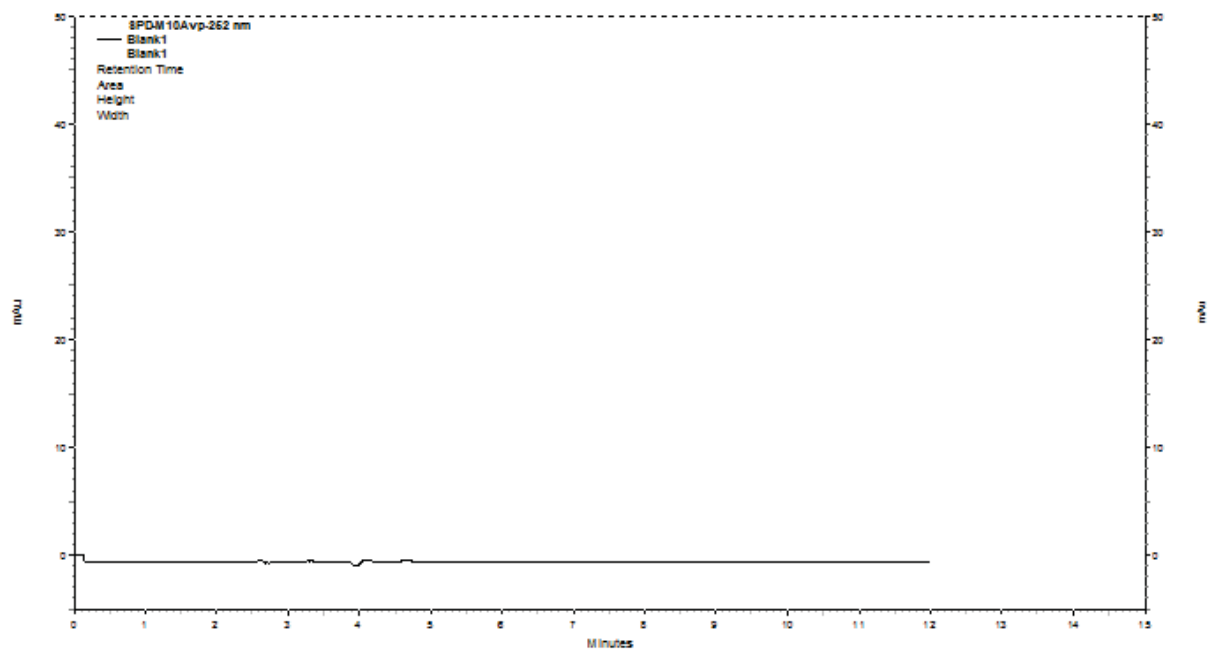
(a)
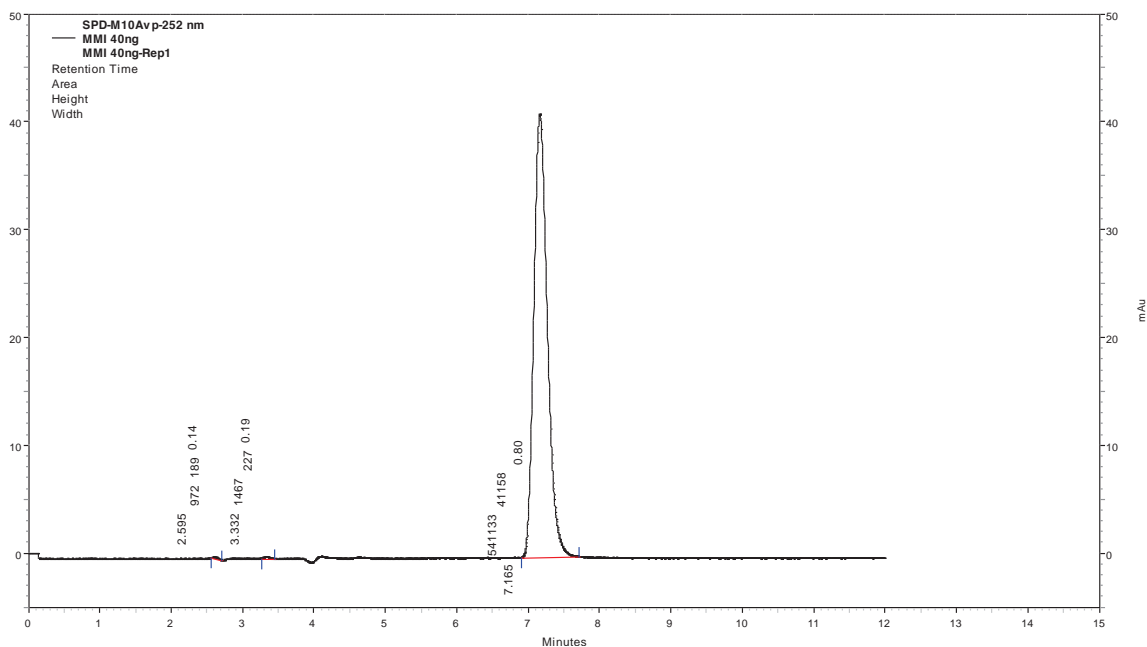
(b)

Figure 2: To determine the specificity of the HPLC, blank plasma from 3 different cats was analysed to check that no other peaks eluted at the same time as methimazole. No interfering peaks were detected in blank samples (a). A methimazole peak is usually seen between 7 to 8 minutes (b).

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Appendix D

Statements of contribution to Doctoral Thesis containing publications

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Kate Hill

Name/Title of Principal Supervisor: Assoc Prof Paul Chambers

Name of Published Research Output and full reference:

Hill KE, Gieseg MA, Bridges J. & Chambers JP: The pharmacokinetics of methimazole in a novel lipophilic formulation administered transdermally to healthy cats, New Zealand Veterinary Journal, 62(4):208-13, 2014

In which Chapter is the Published Work: Chapter 3

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Hill, KE, Gieseg, MA, Kingsbury DD, Bridges J, Lopez-Villalobos, Chambers JP: The efficacy and safety of a novel lipophilic formulation of methimazole for the once daily transdermal treatment of cats with hyperthyroidism. Journal of Veterinary Internal Medicine. 25:1357–1365, 2011.

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Hill K.E., Mills P.C., Jones B.R, Bolwell C.F., Aberdein D, Chambers J.P: Percutaneous absorption of methimazole: an in vitro study of the absorption pharmacokinetics for two different vehicles. Journal of Veterinary Pharmacology and Therapeutics. Accepted January 2015

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Hill K.E., Mills P.C., Jones B.R, Bolwell C.F., Aberdein D, Chambers J.P: Regional variations in percutaneous absorption of methimazole: an in vitro study on cat skin. Journal of Veterinary Pharmacology and Therapeutics. Accepted February 2015

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Name of Published Research Output and full reference:

Hill K.E., Chambers J.P, Jones B.R, Bolwell C.F., Aberdein D, Mills P.C.: Trans-pinnal movement of methimazole: an in vitro study showing that methimazole can cross from the inner to outer pinna of cats. Journal of Feline Medicine and Surgery. doi: 10.1177/1098612X14567548

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