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NOVEL POUR-ON TECHNOLOGY IN CATTLE

**A thesis presented in partial fulfilment of the requirements
for the Degree of Master of Philosophy in Veterinary
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GENERAL ABSTRACT

To date the majority of commercially available pharmaceutical pour-on formulations have been used for the control of internal and external parasites in cattle. The objective of this research was to design pour-ons with novel applications, using cattle as the model species.

Serum and liver α -tocopherol concentrations following topical application of α -tocopherol were investigated using 30 mixed age non-lactating Friesian cows. Cows were randomly allocated to 1 of 5 groups and were treated on day 0 with 1 of 4 formulations or left untreated. Formulations were designed using combinations of permeation enhancers that have been shown to promote the absorption of lipophilic substances. All formulations contained equal quantities of α -tocopherol and were applied at a dose of 0.95 g dl- α -tocopherol acetate / 50 kg live weight. Concentrations of α -tocopherol in serum and liver were monitored for 20 days following treatment. Serum α -tocopherol concentration was significantly increased in 1 group on day 2 and in 2 groups on day 6. It is concluded that α -tocopherol can pass through the skin into the systemic circulation when applied topically.

Thirty Hereford yearlings were used to investigate the concentration of vitamin B₁₂ in plasma and liver following the topical administration of 2 vitamin B₁₂ pour-ons. Animals in the 2 treatment groups received a dose of 6mg cyanocobalamin per 50 kg live weight of their allotted pour-on on the first and seventh days of the trial. Control animals received no treatment. Blood samples were collected from all cows on days 0, 2, 7, 9, and 14 for assay of plasma vitamin B₁₂ concentration. Liver samples were collected from 5 of the cows in each group on days 0, 7, and 14 and were also analysed for vitamin B₁₂ concentration.

Differences between treatment groups in plasma and liver vitamin B₁₂ concentrations were not significant. It was concluded that cyanocobalamin did not cross the skin in high enough concentrations to elicit a statistically significant blood or liver response.

The dermal permeability of 3 selenium pour-on formulations was examined over 24 hours using an *in-vitro* calf skin permeation model. Pour-on formulation A, made up of selenium dioxide and butyl dioxitol, had a statistically greater rate of absorption than formulations B (made up of sodium selenate, butyl dioxitol and water) or C (made up of selenium sesquitrato, butyl dioxitol and water) for the entire 24 hour period. The absorption rates of formulations B and C were not statistically different. The results obtained from the *in-vitro* experiment were then compared with those from an *in-vivo* experiment using the same pour-on formulations, each applied to 6 cattle. Comparison of the 2 data sets indicates that the *in-vitro* model was useful in ranking the formulations in terms of *in-vivo* permeability.

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TABLE OF CONTENTS

General Abstract	ii
Acknowledgements	iv
Table of Contents	v
List of Figures	vi
List of Tables	viii
Chapter 1: Literature Review	1
Chapter 2: Serum and liver concentrations of α-tocopherol in cows following topical application of four vitamin E pour-on formulations	25
Chapter 3: Serum and liver concentrations of vitamin B₁₂ after the application of two vitamin B₁₂ pour-on formulations	36
Chapter 4: <i>In-vitro</i> and <i>in-vivo</i> examination of three selenium pour-on formulations	45
General Discussion	60
References	65

LIST OF FIGURES

Figure 1.1. Possible pathways of penetration of a chemical through intact skin.	6
Figure 1.2. Schematic representation of the two possible pathways of penetration for a chemical diffusing through the intact stratum corneum	7
Figure 1.3. Location of enhancers on a conceptual diagram	14
Figure 2.1. Least squares means (LSM) of serum α -tocopherol for treatment groups A and B and for untreated control group D plotted against sampling dates	31
Figure 2.2. LSM for serum α -tocopherol for treatment groups C and E and untreated control group D plotted against sampling dates.....	32
Figure 3.1. LSM of liver vitamin B ₁₂ concentration (nmol/kg) for yearling cattle treated with a vitamin B ₁₂ formulation (groups A and B) and an untreated control group C.	41
Figure 3.2. LSM of plasma vitamin B ₁₂ concentration (pmol/l) in yearling cattle treated with vitamin B ₁₂ pour-on formulation A or B and an untreated control group C.	42
Figure 4.1. Diffusion cell developed to examine the diffusion of 3 selenium pour-on formulations through cattle skin.	49
Figure 4.2. LSM of selenium concentration in saline for <i>in-vitro</i> cells treated with 1 of 3 selenium pour-on formulations.....	55
Figure 4.3. LSM of serum selenium concentration for cows treated topically with 1 of 3 formulations of selenium, and an untreated control group D, for the first 24 hours..	56

Figure 4.4. LSM of serum selenium concentration for cows treated topically with 1 of
3 selenium pour-on formulations, and an untreated control group D, for days 1 to
60..57

LIST OF TABLES

Table 2.1. Formulations used in the vitamin E pour-on treatment trial28

Table 2.2. Least squares means (LSM) for serum α -tocopherol (mg/l) for cows
treated topically with 1 of 4 formulations of vitamin E, and untreated control
group (group D).30

Table 2.3. LSM for hepatic α -tocopherol concentration (mg/kg) for cows treated
topically with 1 of 4 formulations of vitamin E, and untreated cows (group D)...31

Table 2.4. LSM of skin scores for treatment groups.....32

Table 3.1 Formulation of pour-on treatments A and B40

Table 3.2. LSM of liver vitamin B₁₂ concentrations (nmol/kg) in yearling cattle
treated with vitamin B₁₂ pour-on formulation A or B and an untreated control
group.40

Table 3.3. LSM of blood plasma vitamin B₁₂ concentration (pmol/l) in yearling
cattle treated with vitamin B₁₂ formulation A or B and untreated control group.41

Table 4.1. Formulations used for the *in-vivo* and *in-vitro* selenium pour-on trials.51

Table 4.2. LSM of saline selenium for *in-vitro* cells treated with 1 of 3 selenium
pour-on formulations.53

Table 4.3. LSM of serum selenium concentration for cows treated topically with 1 of
3 selenium pour-on formulations, and an untreated control group D.54

Table 4.4. LSM of albumin concentration for *in-vitro* cells treated with albumin
solution (50 g/l) and an untreated control group.....54

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Thirty-eight years ago, Rogoff and Kohler (1960) demonstrated that the application of 100 ml of a concentrated solution of the organophosphate insecticide, crufomate, to a small area of a cow's skin could control cattle grubs. From this experiment they postulated that the crufomate was absorbed across the skin into the systemic circulation and distributed throughout the body, thus interacting with cattle grubs as they migrate through the tissue. Since this time a number of pharmaceuticals have been formulated to act in the same manner as the crufomate formulation, i.e. a chemical is absorbed through the skin into the bloodstream to result in a therapeutic effect. A formulation of this type is commonly known as a pour-on in the veterinary pharmaceutical industry. The use of pour-ons as a method of drug delivery is attractive to the agricultural industry because:

1. Topical administration is less labour intensive than more conventional methods of drug delivery, i.e. drenching or injecting.
2. A pharmaceutical administered topically does not have to be absorbed through the gastro-intestinal (GI) tract. In the GI tract pharmaceuticals can be extensively metabolised or bound to proteins and other GI tract contents and transit time is unpredictable (Pitman and Rostas, 1981).
3. There is less tissue damage compared to injectable drugs and less trauma compared to both injecting and drenching.
4. The dose can be regulated for single animals more closely than when drugs are presented in feed or as licks.

While pour-ons are now commonplace in the veterinary pharmaceutical market, they are still predominantly used to deliver parasiticides to the systemic circulation of cattle for the control of internal and external parasites. Due to their ease of application when compared to more conventional methods of drug delivery, it would be advantageous to develop pour-ons for therapeutic indications other than parasitism. An example of a pour-on being used in a novel way would be in supplementing vitamins to deficient animals.

In order to develop novel pour-on formulations it is important to review the existing literature on this subject to examine the ways in which these types of products are formulated and how they pass through the skin. This chapter reviews the available literature concerning dermal application of pharmaceuticals. While this thesis is directed toward developing new pour-on formulations for cattle, the literature review covers information relating to a wide variety of applications of dermal products, both human and animal. This is necessary since there is limited literature on pour-on formulations in cattle but there is a large quantity of literature on absorption of pharmaceuticals through human skin.

SKIN STRUCTURE

The skin is the largest organ of the bovine body (Pavletic, 1991), and has the function of protecting the internal organs from extremes in temperature fluctuations, allergens, pollutants, toxic chemicals, and organisms such as bacteria, fungi, parasites, and viruses found ubiquitously in the environment (Riviere and Spoo, 1995). Cattle skin is not uniform in appearance, having specialised areas devoid of hair, e.g. the muzzle and udder, which contrast markedly with the skin of the general body surface. The latter, which has loosely but aptly been termed the 'hairy skin', is characterised by an actively growing coat and extends over most of the animals' body (Jenkinson, 1965). As the pour-ons used in this research will only be applied to the hairy skin, a description of the structure of the hairless skin is not included. Cattle skin is composed of three main parts (Figure 1.1): the subcutaneous fatty layer (hypodermis), the overlying dermis, and the epidermis (the outermost layer of the skin).

THE HYPODERMIS

The hypodermis provides a thermal barrier and mechanical cushion; it synthesises and stores readily available high-energy chemicals (Barry, 1991). It also carries the major blood vessels and nerves to the skin and may contain sensory pressure organs (Williams and Barry, 1992).

THE DERMIS

Cattle skin is 5 to 6 mm thick (Nay and Hayman, 1963), with the dermis making up approximately 80-90 % of this thickness. On a histological level the dermis can be divided into two layers, the papillary and reticular layers. The papillary layer consists of loose connective tissue and connects the epidermis (stratum basal/basal lamina) to the deeper reticular layers of the dermis. The reticular layer consists of dense connective tissue connected to the hypodermis, which is composed mostly of fat. Dispersed throughout both layers of the dermis is a network of arterial and venous blood vessels needed to nourish the cells of the epidermis and dermis as well as take part in the last stages of the percutaneous absorption of chemicals (Riviere and Spoo, 1995). Because the blood supply runs so close to the skin surface it essentially provides sink conditions for most molecules penetrating the skin barrier. The blood supply thus ensures that the dermal concentration of a penetrant is usually near zero, and the resulting concentration difference across the epidermis provides the driving force for transdermal penetration (Williams and Barry, 1992).

Within the dermis there are four structures that form a basic unit termed the hair follicle unit. These four structures are a hair follicle with associated hair, a band of smooth muscle (the arrector pili muscle), a sebaceous gland, and a sweat gland. In cattle, hair follicle units are situated apparently at random and display no obvious pattern of distribution (Jenkins, 1965).

THE EPIDERMIS

The following explanation of the structure of the epidermis was derived from Riviere and Spoo (1995). The epidermis consists of two primary cell types, those of keratinocyte origin and those of nonkeratinocyte origin. Five distinct layers of keratinocytes can be present in the epidermis. From deepest to most superficial they are (1) stratum basale; (2) stratum spinosum; (3) stratum granulosum; (4) stratum lucidum (found only in areas of very thick skin); and (5) stratum corneum. Each cell layer has its point of origin at the stratum basale which is a single layer of cuboidal or columnar cells that rests on the basal lamina.

The stratum basale cells continuously divide and move toward the skin's surface although some remain as basal cells. As they move outward they change their intracellular content by a process called keratinization. This is how the five separate layers are formed. Each group of cells has a different make up. The stratum spinosum has irregularly shaped polyhedral cells and these make up the bulk of the thickness of the epidermis. The stratum granulosum consists of several layers of cells that begin to flatten horizontally. There are laminated granules within these cells that contain polar phospholipids such as glycosphingolipids and free steriods and numerous hydrolytic enzymes. As these intracellular products accumulate they are exocytosed by the cell to fill the intercellular space. Eventually they form the extracellular lipid matrix of the stratum corneum.

The stratum corneum is the final and most superficial layer of the epidermis and is the most important layer of the epidermis when considering the success or failure of topical drug therapy because it is the primary barrier to percutaneous absorption. The stratum corneum consists of several layers of dead cells, organised into vertical columns in a tetrakaidecahedral (14-sided) configuration, the thickness of which depends on location. Each cell is ingrained in the lipid matrix produced by the laminated granules when the cells were still in the stratum granulosum. These dead cells are also surrounded by a thick plasma membrane with a submembranous layer of involucrin also produced earlier in development.

Due to its make-up, the stratum corneum discourages the passage of chemicals and toxins from the external environment passing through the skin. Unfortunately the skin does not distinguish between unwanted chemicals and therapeutic pharmaceuticals.

PATHWAYS OF PENETRATION

Two general pathways exist for a molecule to penetrate normal intact skin: via the skin appendages (hair follicles and sweat glands), together known as the appendageal route, or across the intact epidermis (Williams and Barry, 1992) as shown in Figure 1.1.

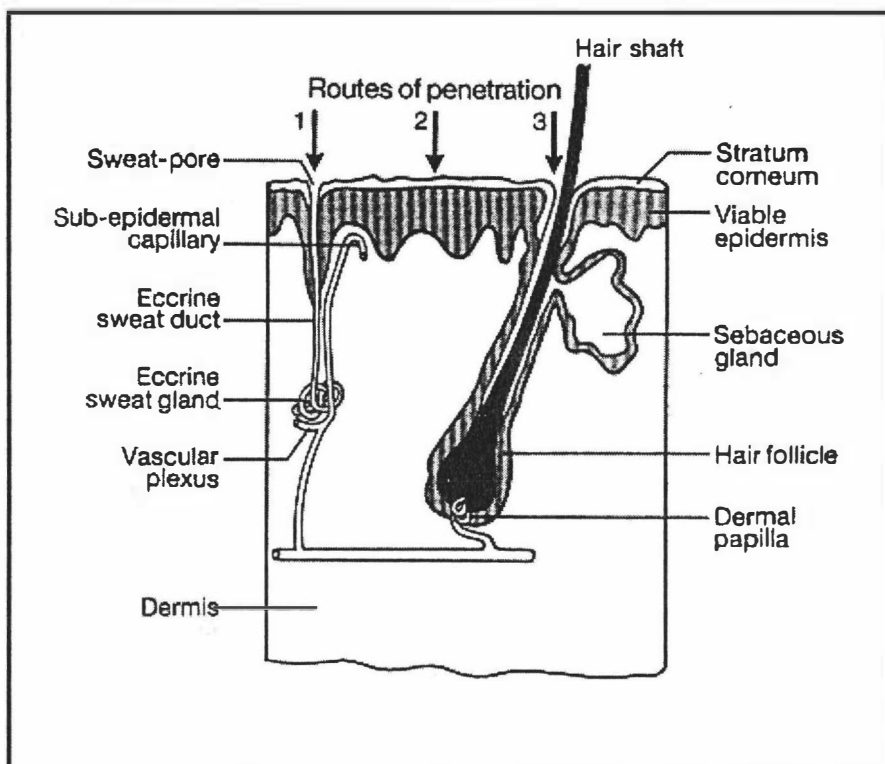


Figure 1.1. Possible pathways of penetration of a chemical through intact skin. Arrows 1 and 3 point to the appendageal route. Arrow 2 points to the epidermal route (Williams and Barry, 1992).

THE EPIDERMAL ROUTE

For chemicals whose main pathway of penetration through the epidermis is to first pass through the stratum corneum, it has been suggested that there are two possible portals of access, the intercellular and transcellular paths (Figure 1.2). The transcellular pathway involves the drug passing through the stratum corneocytes and associated extracellular lipid matrix, then through the cells of the deeper epidermis and finally into the systemic circulation. The intercellular pathway involves the drug manoeuvring its way through the stratum corneum via the extracellular lipid matrix only.

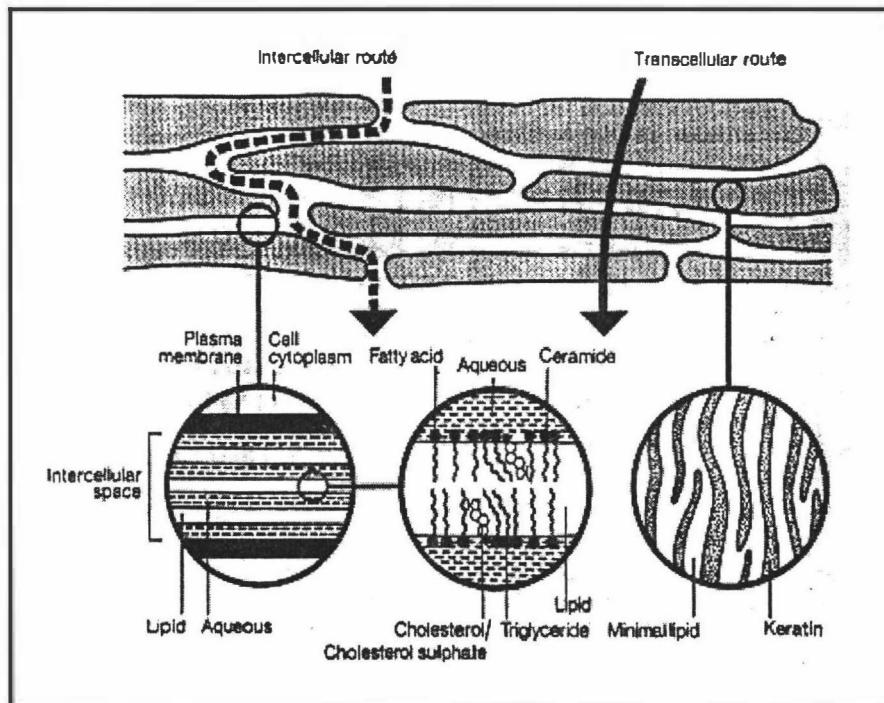


Figure 1.2. Schematic representation of the two possible pathways of penetration for a chemical diffusing through the intact stratum corneum (Williams and Barry, 1992).

When a drug permeates through the epidermis, no matter which of the two pathways it takes, it will always have to pass through the lipid matrix that holds the stratum corneocytes together. Therefore it would be assumed that hydrophilic molecules would have low diffusivity through the skin as they would be unable to diffuse through this lipid barrier. This is not the case; the absorption of hydrophilic molecules has been demonstrated by a number of authors (Hori *et al.*, 1973; Cornwell and Barry, 1994; Ogiso *et al.*, 1994; Hilton *et al.*, 1994)

Flynn (1989) proposes that aqueous pores through the corneocytes may act as a channel for the penetration of highly hydrophilic molecules. In order for the molecule to pass to the next corneocyte it must then pass through the intercellular lipid matrix. Flynn proposes that the hydrophilic molecules pass through the lipid matrix via lacunae. Lacunae are discontinuities or defects within the intercellular lipid matrix. The drug passes from one corneocyte to the other via lacunae until it reaches the viable epidermis. From there it transverses through the dermis to the systemic circulation.

In contrast to this theory, other authors (Potts and Francoeur, 1992; Potts *et al.*, 1992) have suggested that hydrophilic molecules pass through the stratum corneum not via aqueous pores through the corneocytes, but by way of kinks in the lipid alkyl chains of the lipid matrix. It is further proposed that it is the small size and molecular weight of many hydrophilic molecules that aid their transport through the lipid alkyl chain kinks. This theory is supported by the fact that larger hydrophilic molecules have lower absorption than their smaller molecular weight counterparts (Kasting *et al.*, 1987).

APPENDAGEAL ROUTE

Skin appendages bypass the low diffusivity of the stratum corneum and may act as diffusional shunts. This pathway of penetration in human skin is widely believed to provide an insignificant pathway for most drug permeation (Blank, 1964; Scheuplein, 1971) as the appendages only make up approximately 0.1% of the total skin area. Skin appendages in cattle take up a far greater amount of the total surface area of the skin. While a square centimetre of human skin contains 10 hair follicles, 15 sebaceous glands and 100 sweat glands (Lubowe, 1963; Wells, 1964), the same area of cattle skin contains approximately 2000 hair follicles with associated sweat and sebaceous glands and ducts. Therefore it seems logical that appendageal transport could play a more important role in the transport of chemicals through cattle skin. Appendages in human skin, though few in number, may play a major role in the absorption of ions (Scheuplein, 1976) and large polar molecules (Wahlberg, 1968). The density of the appendages becomes even more significant when considering the absorption of those chemicals through cattle skin.

Pitman and Rostas (1981) propose that the rate and extent of drug absorption across cattle skin is significantly influenced by the composition and physical properties of the sebum-sweat emulsion associated with the skin and skin appendages. They suggest that the appendageal pathway is likely to be the main pathway of penetration through cattle skin, with the sebum-sweat emulsion associated with the appendages being the main barrier to penetration.

THE DIFFUSION PROCESS

Although the mechanisms for drug transport across the skin have not yet been fully elucidated, it is clear that a process of passive diffusion occurs (William and Barry, 1992). In general, absorption follows Fick's first law of diffusion:

$$\frac{dQ}{dt} = \frac{(PC)C_vDA}{h}$$

where dQ/dt is a steady rate of penetration, PC is the drug's partition coefficient between the vehicle and the stratum corneum, C_v is the concentration of the drug dissolved in the vehicle, D is the diffusion coefficient, A is the area of the skin to which the drug is applied, and h is the diffusion path length through the stratum corneum. Fick's law states that the driving force causing the transfer of a drug from areas of high concentration to areas of low concentration is proportional to the concentration gradient (Idsen, 1975). While this formula may hold true for a number of chemicals crossing human skin it needs to be modified if it is to represent the passage of diffusion through cattle skin. Pitman (1981) reported that while the stratum corneum was the rate limiting barrier to the absorption of levamisole through human skin, the rate of penetration of levamisole through cattle skin was dependent on total skin thickness. When considering the above equation in relation to the absorption of specifically levamisole, and perhaps other chemicals through cattle skin, it may be more appropriate to include the total skin thickness rather than the stratum corneum thickness.

Pitman and Rostas (1981) have also suggested that the following equation can be used if molecules diffuse through the skin appendages and these present a homogeneous barrier, i.e. if the appendageal pathway predominates.

$$J = \frac{DaKa}{Ta} \Delta C_s = kp.a \Delta C$$

where J is the flux, Da is the diffusion constant of penetrant molecules in appendages, Ka is the appendage/vehicle partition coefficient of penetrant, Ta is the thickness of appendages that must be traversed, ΔC_s is the external concentration difference, and $kp.a$ is the permeability constant of penetrant molecules in appendages.

PARTITIONING COEFFICIENT AND DIFFUSIONAL COEFFICIENT

The partition coefficient is a unitless measure of the relative affinity of a compound between a highly hydrophobic phase (usually octanol) and a hydrophilic phase (water), and is a physiochemical property that has some role in determining the ability of a chemical to penetrate the skin (Riviere and Spoo, 1995). A large partition

coefficient value indicates that the vehicle has poor affinity for the drug. A low partition coefficient value reflects the tendency of the drug to remain in the vehicle (Idson, 1983). In general examining the partition coefficient of a number of formulations can help in deciding which one will penetrate the skin more readily. The exception to the rule is steroids. The ability of a steroid to permeate the skin can not be related to the partitioning coefficient, instead it can be related to the diffusion coefficient. The diffusion coefficient reflects the ease with which the molecules move through the various membrane strata. Because steroids can have a high degree of chemical interaction with the lipid matrix of the stratum corneum, the diffusion coefficient is a much better measure of how well a steroid will penetrate the skin.

POUR-ON FORMULATION

A pour-on formulation usually contains three main components: 1) penetrant(s); 2) vehicle; and 3) penetration enhancer(s). The penetrant is the drug or chemical that is being delivered to the systemic circulation to have some sort of therapeutic effect. The vehicle can be defined as the medium in which a medicinally active agent is topically administered (Riviere and Spoo, 1995), and is predominantly water or oil in many of the commercial pour-on formulations currently available. A penetration enhancer is a chemical that is included in a formulation to promote the absorption of the penetrant through the skin. Some pour-on formulations contain only a vehicle and a penetrant. In this case the vehicle often acts as the penetration enhancer.

PENETRANT

The penetrant is usually the easiest component of a pour-on formulation to select because there will generally be a limited number of chemicals that will produce the therapeutic effect desired on entering the systemic circulation. As a general rule most formulation designers decide what they want to cross the skin and then go about designing a formulation that will get the penetrant across the skin. As with the vehicle and enhancer, the penetrant should be non-toxic and non-irritant.

VEHICLE SELECTION

The physical and chemical properties of the vehicle can be related to how successfully a chemical will cross the skin. When selecting a vehicle for a pour-on formulation the following characteristics are desirable:

- The vehicle should provide a balance that partitions the drug between the vehicle and the skin, so that the drug has a significant degree of affinity for the vehicle but still attains adequate delivery from the vehicle to the skin (Higuchi, 1960; Barr, 1962).
- The vehicle should not be an irritant or harm the skin and in some cases may even have some protective or therapeutic effects on the applied site itself (Oishi, 1975).
- The vehicle should be a suitable solvent for the drug. Including an enhancer in the formulation may increase the ability of a drug to dissolve in that vehicle.
- Preferably the vehicle should be inexpensive as it usually makes up the largest proportion of a pour-on and can therefore greatly affect the price.
- From a commercial point of view the vehicle should also be cosmetically acceptable.
- The vehicle should wet down well when applied to the skin surface and not hold up in the hair or run off the skin. Some pour-on formulations include a wetting agent in the vehicle.

PENETRATION ENHANCERS

When selecting a penetration enhancer for a pour-on formulation it would ideally meet all of the requirements suggested for the vehicle as shown above. Some other attributes of an ideal enhancer may be listed as follows:

- The barrier properties of the skin should reduce in one direction only. Endogenous materials should not be lost to the environment by diffusion out of the skin (Barry, 1991).
- Upon removal of the enhancer, the skin should immediately and fully recover its normal barrier properties.
- The material should be pharmacologically inert.

While all the above properties for both the vehicle and the penetration enhancer are desirable in a pour-on formulation it is unlikely that you would find chemicals that possess them all. Researchers designing products for topical application therefore try to select chemicals that meet as many of the requirements as possible.

Mode of action of penetration enhancers.

The lipid-protein-partitioning concept is a theory that has been proposed to explain the mode of action of all the known penetration enhancers, including penetration via the intercellular as well as the transcellular routes (Barry, 1991). Barry (1991) suggests that all penetration enhancers may act by one or more of three main mechanisms:

1. Disruption of the highly ordered structure of the stratum corneum lipids.
2. Interaction with intercellular protein.
3. Improved partitioning of a drug, coenhancer, or cosolvent into the stratum corneum.

Examples of all the above mechanisms have been cited in other publications. Goodman and Barry (1985, 1986) found azone reduced the order of the intercellular lipid matrix. Williams and Barry (1991), when using terpenes, saw a similar effect. Other enhancers such as nonionic surfactants and the keratolytic chemical urea, interact with the intracellular protein component of the stratum corneum (Williams and Barry, 1992). Small polar enhancers such as dimethyl sulfoxide (DMSO) and its analogues, and the pyrrolidones, propylene glycol and ethanol may accumulate in the horny layer and promote increased partitioning of the drug out of the vehicle into the skin (Barry, 1991).

Penetration enhancer classification

Hori, Satoh and Maibach (1989) proposed a classification of penetration enhancers using a conceptual diagram (Figure 1.3). This diagram was originally developed to predict the properties of organic compounds (Fujita, 1954). Assigning an organic and inorganic value for a given compound depending on its structural components allows derivation of the diagram. The organic parameter depends on the number of carbon atoms and the inorganic parameter depends on the number of substituted groups. An

organic value is derived by summing up the number of carbon atoms in a compound, with each carbon atom being assigned a predetermined value. An inorganic value is derived by summing up the number of substitution groups, with each substitution group being assigned a predetermined value. The inorganic and organic values for a given compound can be found in the text (Fujita, 1954; Kouda, 1984). Once inorganic and organic values are known they are plotted on a graph to derive the conceptual diagram. The diagram has been used to divide enhancers into three different areas that represent their different physiochemical properties. Area I contains enhancers that are solvents and includes many of the “classical enhancers” such as 2-pyrrolidone, dimethyl sulfoxide and propylene glycol. Area II contains enhancers for hydrophilic compounds such as azone and oleic acid. Area III contains enhancers for lipophilic compounds such as pinene and limonene. It should be noted that the areas in the diagram are arbitrary and some enhancers can be classified in more than one of the areas, for example azone has been shown to promote the absorption of both hydrophilic and lipophilic compounds. While this is the case, this diagram is still very useful to refer to as a starting point when trying to decide on enhancers to use in a particular pour-on formulation.

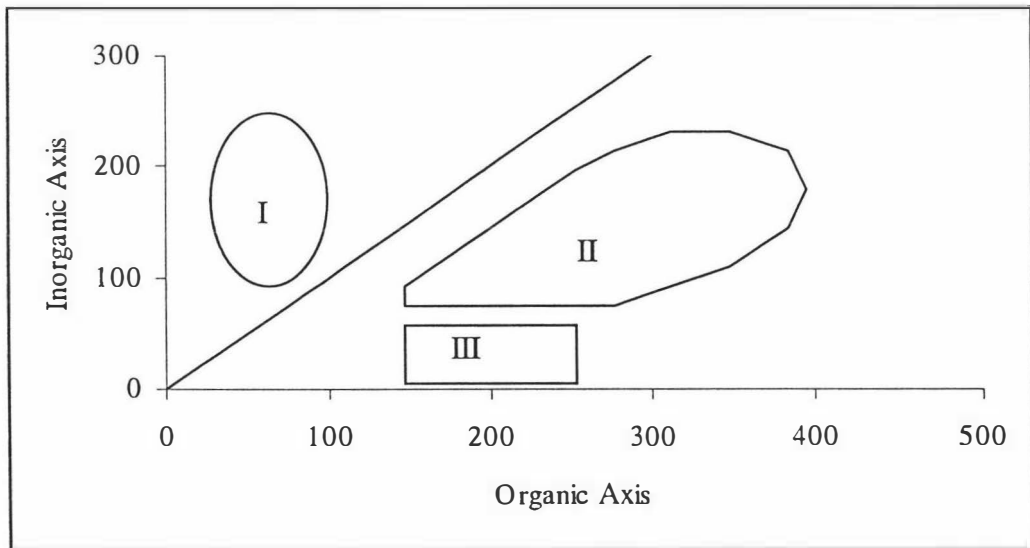


Figure 1.3. Location of enhancers on a conceptual diagram (Hori *et al.*, 1989). Area I, enhancers which are solvents; Area II, enhancers for hydrophilic compounds; Area III, enhancers for lipophilic compounds.

FACTORS AFFECTING POUR-ON ABSORPTION

The physical and chemical properties of the vehicle, as well as skin, drug and vehicle interactions, can influence the permeation process and the therapeutic efficacy of topical products (Twist and Zatz, 1988). There are two main rate-determining effects in pour-on absorption, the effects due to the skin and the effects due to the pharmaceutical.

RATE DETERMINING EFFECTS DUE TO THE SKIN

The amount of pharmaceutical the skin will absorb depends on the anatomical site to which it is applied. Qiao (1993) investigated the effects of dosing site on the absorption of parathion *in-vivo* in pigs. Four sites were tested: abdomen, buttocks, back and shoulder. Results showed that the order of absorption appeared to be abdomen<buttocks<shoulder<back. Absorption from shoulder and back were significantly higher than that of abdomen and buttocks. Numerous authors have shown variation in absorption due to the site of application (Wester *et al.*, 1984; Feldmann and Maibach, 1967; Wester *et al.*, 1980)

Regional differences in absorption rates may be related to skin thickness, the number of cell layers and cell size of the epidermis and stratum corneum, the distribution of hair follicles, sweat and subcutaneous glands. Anatomical structure and physiological functions of the cutaneous microcirculation as well as the extent of keratinization, the rate of stratum corneum shedding and cutaneous biotransformation may affect the absorption process also.

The function of the skin is altered by changes in environmental conditions which can affect the rate at which pharmaceuticals are absorbed. There were a number of papers in the early 1980's that looked at the effect of season on the absorption of the anthelmintic levamisole in cattle. Forsyth *et al* (1983) presented a paper that showed the results from a number of experiments conducted over a three year period in Australia. In all experiments the blood serum concentration of levamisole was determined for a number of hours after application of the pour-on. Then each trial animal was slaughtered three to seven days after treatment and residual worm burdens

were determined. These results were then compared with those following a subcutaneous injection of levamisole. A total of six experiments were carried out. The first two experiments conducted in July and August of 1978 (cold months) gave very poor anthelmintic effect and blood serum concentrations, both of which were inferior to that of the subcutaneous injection. This suggested that a higher dose rate should be used in the colder months. Experiments three and four were conducted in the warmer months of March and November of 1979. Results showed an increase in anthelmintic activity and blood serum concentration at the same dose rates used in the first two experiments. This suggested that seasonal temperature has an influence on dermal absorption. Experiment five conducted in May 1981 (mild weather) showed the pour-on to have the same anthelmintic activity as the injection but a lower levamisole blood serum concentration. Experiment six conducted in July 1982 (cold month) confirmed the poor efficacy of dermally applied levamisole under cold conditions.

The researchers concluded from the above experiments that there was an obvious influence of season on the dermal absorption of levamisole, and that this difference seemed to be due to temperature.

Pitman and Rostas (1981) performed *in-vitro* studies on the penetration of levamisole through excised cattle skin. They reported that the drug penetrated 10 times faster through skin harvested in summer than skin harvested in winter. This study postulates that the seasonal changes in permeability are due to seasonal changes in the composition of emulsion on the surface of the skin that result from seasonal changes in sweat and sebum.

Forsyth (1984) studied the effect of coat length on the bioavailability of levamisole applied topically to cattle. The experiment involved 20 cattle split into four groups. The first group had the hair on their back clipped 24 hours before the treatment. The second group remained unclipped. Both groups were treated with a levamisole pour-on at a rate of 20 mg/kg body weight. The third group was given a subcutaneous injection (6 mg/kg body weight) while the fourth group received no treatment. Results showed that the serum concentrations of levamisole were significantly higher in the injected group than the other groups. Concentrations of levamisole in serum were not

significantly affected by clipping hair. This experiment indicated that the longer winter coat of the cattle does not seem to be a factor that influences the amount of percutaneous absorption. In fact the clipped skin was probably colder than the unclipped skin and this may have reduced absorption. If this were the case, the effect of the skin temperature may have obscured effects due to coat length.

Taylor *et al* (1983) performed a study that looked at the variation in bioavailability of topical levamisole in cattle relative to season and ambient temperature. Nine trials were conducted. Four were carried out in winter months (October to March, mean ambient temperature 4.7 degrees) and five in the summer months (April to September, mean ambient temperature 14.4 degrees). All trials tested levamisole serum concentration at set time intervals after the application of the pour-on. Results showed that bioavailability was 60 to 70 % higher in summer than in winter. This author proposes that the different absorption rates are due to seasonal changes in the skin structure rather than differences in ambient temperature, although he does say that changes in ambient temperature may have a minor effect on absorption.

Seibert *et al* (1986) looked at the seasonal anthelmintic activity of levamisole pour-on in cattle in the USA. Two main trials were carried out. In the first one (a controlled efficacy trial) levamisole-pour-on was administered at a rate of 10 mg/kg body weight to 18 cattle in the summer with 18 untreated cattle used as controls. The same treatment was applied in winter to two groups of 16 cattle. Seven to nine days after treatment the animals were sacrificed and adult worm burden was recorded. In the second trial (a field investigation) veterinarians from nine different geographic locations selected 1302 healthy beef cattle to be included in a field comparison of summer and winter efficacy of levamisole pour-on. Trials were conducted on a number of different farms using levamisole pour-on and a placebo pour-on as a control. Results were obtained by analysing faecal samples for the presence of nematode ova at the time of treatment and one week later and by measuring ambient temperature during treatment application. Results from the first trial showed that there was no difference in the anthelmintic efficiency of the levamisole pour-on in the two seasons tested. Similarly, results from the second trial showed no significant difference in the efficacy of levamisole pour-on based on ambient temperature at the

time of application. This paper disagrees with the earlier mentioned papers by showing that there is no significant difference in dermal absorption of levamisole at different ambient temperatures and in different seasons.

Chang *et al* (1994) studied the effect of a combination of environmental parameters on the percutaneous absorption of an agricultural pesticide by the pig *in-vitro* using a two chambered Teflon flow through diffusion cell. Environmental parameters were varied using an air temperature and humidity control unit. They found that knowledge of the effect of a single environmental variable on the absorption profile of a topical penetrant is not sufficient to estimate its absorption in an environment where multiple variables are changing independently.

RATE-DETERMINING EFFECTS DUE TO THE PHARMACEUTICAL

The makeup of the pharmaceutical used will affect its potential to be absorbed. When considering the rate at which a pharmaceutical can be absorbed two main factors need to be taken into account: (1) the type of substance required to cross the skin; and (2) the vehicle to be used to get the substance across the skin.

Factors relating to substance to be absorbed

Small uncharged molecules are relatively easily absorbed through the skin by passive diffusion. Large charged molecules such as peptides and proteins are more poorly absorbed through the skin due to their large molecular weights and radii. Iontophoresis has been found to provide a method for the transdermal delivery of large, charged molecules that cannot be delivered passively. Iontophoresis has been demonstrated by a number of authors (Heit, 1993; Guy 1992; Banga and Chien 1988; Siddiqi *et al* ., 1987) and in its most simple form is the driving of charged molecules into tissue using an electrical current. Other methods of promoting absorption of molecules that will not be absorbed passively are phonophoresis (Camel, 1995; Griffin and Touchstone, 1963; Novak, 1964; Tyle and Agrawala, 1989) and electroporation (Chang and Reece, 1990; Weaver,1993). These methods would be of little practical use in the development of a pour-on for administration to animals as they are more time consuming and expensive than the methods already in place for getting drugs into the systemic circulation.

The concentration of the applied dose, the surface area of the site, and the elapsed time the chemical is on the skin are the main variables affecting absorption. As the concentration of the drug is increased, the total amount absorbed increases until the skin is saturated. Increasing the surface area of the applied dose also increases absorption. Absorption occurs over time so the longer the substance is on the skin, the greater the chance of continued absorption.

The vehicle

The ability of a vehicle to stay on the skin to allow the absorption of the drug is another important factor in drug absorption. The vehicle can be removed by three mechanisms: (1) absorption of the vehicle through the stratum corneum; (2) evaporation from the skin surface into the surrounding air; or (3) physical removal (rubbing, licking, scratching, etc.).

If the vehicle permeates through the skin more quickly than the drug, then there may be a precipitation of the drug on the skin surface that may not be absorbed. The evaporation of the vehicle into the surrounding environment could have a similar effect. The vehicle may also in some way change the permeability of the lipid matrix, perhaps making it more susceptible to penetration of the drug.

Vehicles may also effect the hydration state of the stratum corneum. This can affect the rate at which drugs will pass through it. Increasing the hydration state of the stratum corneum will increase the rate at which a drug will permeate it. An increase in percentage hydration from 10 to 50 % in the stratum corneum can result in as much as a 10-fold increase in diffusion constants (Idson, 1983). Conversely, a decrease in the hydration of the stratum corneum can decrease the percutaneous absorption of substances.

SKIN PERMEATION MODELS

Once a pour-on formulation has been developed, it needs to be tested. This can be done on live animals, but it is advantageous in terms of cost and efficiency to test products in the development stage using a model. *In-vitro* experiments using a model can be carried out with much less effort and in greater numbers because of the simplicity in methodology. They also do not have welfare implications and do not damage marketable hides.

TYPES OF *IN-VITRO* MODELS

A number of *in-vitro* models have been used to test if a compound is likely to penetrate the skin. In many of the earlier absorption studies two chambered diffusion cells were used. These consist of a piece of skin sandwiched between two cells. One cell containing a receptor solution and the other a donor solution. The two chamber diffusion model is still used today in studies where steady-state kinetics are important. The one chambered cell is used to study absorption under more normal exposure conditions. The one chamber diffusion model consists of one cell that holds the receptor fluid and is beneath the skin. The method allows the test compound to be applied in the vehicle of choice without affecting the hydration state of the skin.

The type of fluid used in the receptor phase will affect the results of any permeation experiment. If necessary, viability of the skin can be maintained by using a physiological receptor fluid. If this is the case best results are obtained by the continual replacement of this fluid with a flow through cell (Bronaugh 1989). The choice of what receptor fluid to use in the receptor chamber should be related to the type of penetrant that is being examined. For water soluble penetrants an aqueous receptor solution can be used. For hydrophobic penetrants a lipophilic receptor fluid should be used. If an inappropriate receptor fluid is used the penetrant may have low tendency to partition into the receptor fluid.

Regardless of the type of cell used the preparation of the skin is a critical step in permeation models. If full thickness skin is used in diffusion cells studies, the thick

dermal tissue can present an artificial barrier. Compounds that are absorbed through the skin *in-vivo* are taken up by the blood vessels directly beneath the epidermis, so they are not required to penetrate the full thickness of the skin (Bronaugh 1989). To overcome this problem a dermatone can be used to remove the majority of the dermis.

It is not necessary to always use excised skin as the permeation barrier in diffusion models. It is possible to use synthetic membranes that have similar properties to the stratum corneum. Synthetic membranes are advantageous over skin samples as they require less stringent handling conditions, can be obtained in a variety of forms and thickness, and are less variable allowing small changes in permeability to be detected.

A number of authors have shown a good relationship between *in-vivo* and *in-vitro* measures of absorption. Aimsworth (1960) observed a similarity in tributyl phosphate penetration through rabbit and pig skin using excised and intact living skin. Identical *in-vivo/in-vitro* absorption was obtained through the stratum corneum of rabbit ear for water and tripropyl phosphate (Creasy *et al.*, 1978). Bronaugh *et al* (1982) compared the absorption of three compounds with differing permeability properties through excised and intact rat skin. The *in-vitro* absorption was nearly identical to the *in-vivo* absorption.

COMMERCIALLY AVAILABLE POUR-ON PRODUCTS FOR CATTLE

The pour-on treatments listed below are examples of products designed for absorption into the systemic circulation to have a therapeutic effect. There are other pour-ons in the pharmaceutical market that are used to control external parasites and are not designed to enter the systemic circulation. These products are not mentioned below.

Anthelpor: Contains 200 g/L levamisole.

For control of gastro-intestinal roundworms and lungworms in cattle.

Bomatak white-strip pour-on:

Contains 75 g/L oxfendazole

For the control of gastro-intestinal round worms, lungworms and tapeworms in cattle.

Eprinex pour-on for cattle and deer:

Contains 5 mg/ml eprinomectin.

For the treatment and control of a broad range of internal and external parasites of beef and dairy cattle (including lactating cows) and internal parasites of deer.

Genesis pour-on:

Contains 10 mg/ml abamectin.

For the control and treatment of internal and external parasites in cattle.

Ivermec pour-on:

Contains 0.5% w/v ivermectin

For the treatment and control of a number of species of gastrointestinal roundworms, lungworm, lice, mange mites, cattle grubs and ticks.

Selpor:

Contains 5 mg/ml sodium selenate

For the prevention and treatment of selenium deficiency and selenium responsive diseases in cattle.

Vetdectin pour-on for cattle and deer:

Contains 0.5% w/v moxidectin.

For the treatment and control of a broad range of internal and external parasites in cattle, and the treatment and control of lungworms and roundworms in deer.

PROPOSED RESEARCH

DEVELOPMENT OF POUR-ONS FOR USE IN VITAMIN AND TRACE ELEMENT SUPPLEMENTATION

Considerable evidence exists that vitamins can be administered topically and absorbed into the systemic circulation. Sobel and Rosenberg (1953) stated that topical application of vitamin A was nearly as effective in overcoming vitamin A deficiency in rats as the oral route. Schaefer (1955) and his fellow authors tested the absorption of a number of different vitamins (thiamine, riboflavin, pantothenic acid, pyridoxine and vitamin D) through the skin of rats. The absorption of the topically applied vitamin was measured by the excretion in the urine and the animal's increase in body weight. From this study it was found that all of the vitamins tested were able to be absorbed across the skin. This was demonstrated by body weight gain responses and the prevention or cure of deficiency symptoms for each of the different vitamins tested.

From this information and the other information in the above review on pour-on formulations it appears that a pour-on could be designed that provides vitamin and/or trace element supplementation for cattle (Chapter 2 and 3). Inclusion of vitamins and trace elements in anthelmintic pour-ons would be advantageous and allow pour-ons the same marketing advantage as oral formulations which contain nutrients. Vitamin E and B₁₂, selenium and copper are of the most interest to the NZ agricultural industry.

SEASONAL VARIATION IN ABSORPTION OF ANTHELMINTICS

There is contradictory evidence in relation to whether temperature effects the permeability of levamisole. For example, the different results from the Australian (Forsyth *et al.*, 1983) and American (Seibert *et al.*, 1986) trials, and the proposal by Taylor *et al.* (1983) that the changes he found to occur in permeability between summer and winter applications of levamisole were due to seasonal changes in skin structure.

It would be interesting to perform controlled experiments in NZ to test whether

variation in temperatures and season affect the rate of absorption of topical anthelmintic and trace element products.

A MODEL FOR ABSORPTION

Setting up an *in-vitro* model to examine the degree to which chemicals cross the skin may allow more formulations to be tested in a shorter period of time. Welfare and economic concerns from potential skin or coat damage will not be a feature of *in-vitro* experiments.

Yazdanian (1995) performed *in-vitro* experiments using cattle skin as a membrane and levamisole as a penetrant. Setting up a model using Yazdanian's methodology seems the most appropriate step in testing cattle pour-on formulations *in-vitro* (Chapter 4).

**CHAPTER 2: SERUM AND LIVER
CONCENTRATIONS OF α -TOCOPHEROL
IN COWS FOLLOWING TOPICAL
APPLICATION OF FOUR VITAMIN E
POUR-ON FORMULATIONS**

ABSTRACT

Serum and liver α -tocopherol concentrations following topical application of α -tocopherol were investigated using 30 mixed age non-lactating Friesian cows. Cows were randomly allocated to 1 of 5 groups balanced for live weight and were treated on day 0 with formulations 1 to 4 or left untreated (control). Formulations contained combinations of permeation enhancers that have been shown to promote the absorption of lipophilic substances. All formulations contained equal quantities of dl- α -tocopherol acetate and were applied at a dose of 0.95 g dl- α -tocopherol acetate / 50 kg live weight. Concentrations of α -tocopherol in serum and liver were monitored for 20 days following treatment. Serum α -tocopherol concentration was increased in 1 group on day 2 and in 2 groups on day 6. It is concluded that α -tocopherol can pass through the skin into the systemic circulation when applied topically.

INTRODUCTION

Vitamin E is a biological antioxidant. It is found in lipids and protects adipose tissue and cell membranes from oxidant damage (Naylor and Ralston, 1991). Adequate vitamin E in ruminant diets is important for the prevention of deficiency diseases, such as nutritional myodegeneration (Hidiroglou *et al.*, 1972), and in the protection of milk from spontaneous oxidation (St-Laurant *et al.*, 1990). Injection of vitamin E in conjunction with selenium has been shown to decrease the incidence of retained placenta in overseas studies (Julien *et al.*, 1976; Trinder *et al.*, 1973). Dairy heifers supplemented with vitamin E in typical calf diets show greater weight gains than unsupplemented calves (Reddy *et al.*, 1985). If a ruminant receives inadequate vitamin E in its diet then the vitamin needs to be supplied from another source to maintain health and productivity. Current routes of administration include oral drenching, dietary supplementation or injection of the vitamin. The objective of this trial was to examine the effectiveness of a pour-on formulation as a novel method of supplying vitamin E to the systemic circulation.

MATERIALS AND METHOD

A group of 30 mixed age non-lactating Friesian cows were used. The mean weight was 410 kg at the start of the trial. The cows were fed a diet of 90 % ryegrass and clover hay and 10 % ryegrass and clover pasture for 4 weeks before the trial and remained on this diet for the entire trial. On the first day of the trial (day 0) each cow was assigned randomly to 1 of 5 groups of 6 animals balanced for live weight. Groups were labelled A, B, C, D and E. Group D was randomly selected as the control group and received no treatment. Groups A, B, C, and E were assigned pour-on treatments 1, 2, 4, and 3, respectively (see Table 2.1 for the formulation of the pour-on formulations applied). On day 0 a blood sample was obtained from every cow and a liver sample was obtained from 3 cows in each group. Blood samples were obtained from all cows again on days 2, 6, 14, 20, and 27. The cows that were liver sampled on day 0 were also liver sampled on days 6 and 20. After the samples were taken on day 0 the pour-

on formulations were applied to the cows in groups A, B, C, and E. All treatments were applied as a single strip, directly along the unprepared skin of the back. The formulations were applied using a syringe at a dose rate of 3ml/50 kg of body weight. This gave a dose of 0.95 mg dl- α -tocopherol acetate/ 50 kg live weight. On each sampling day, the midline of the back was examined and scored using a scale of 1 (discoloration or hair loss over the total application site) to 5 (no discoloration or hair loss) to record any discoloration or hair loss.

Table 2.1. Formulations used in the vitamin E pour-on treatment trial

		<u>%w/v</u>	<u>Group</u>
<u>Pour-on 1</u>	isopropyl myristate	40	A
	vitamin E	31.5	
	sesame oil	28.5	
<u>Pour-on 2</u>	octanol	10	B
	vitamin E	31.5	
	sesame oil	58.5	
<u>Pour-on 3</u>	tween 80	25.0	E
	benzyl alcohol	2.0	
	vitamin E	31.5	
	butyl dioxitol	20.0	
	water	21.5	
<u>Pour-on 4</u>	octanol	10.0	C
	isopropyl myristate	20.0	
	vitamin E	31.5	
	sesame oil	38.5	

SAMPLE COLLECTION AND METHODS OF ANALYSIS

Blood samples were obtained via caudal venipuncture and were collected into plain evacuated plastic tubes. Tubes were kept under refrigeration at 4°C for 24 hours then centrifuged and the serum decanted. Samples were then submitted to the Ministry of Agriculture and Fisheries, Wallaceville for α -tocopherol analysis. Concentration of α -tocopherol was measured using normal phase high performance liquid chromatography (HPLC) (Rammel *et al.*, 1983; Rammel and Hoogenboom, 1985). Samples were prepared for HPLC by sponification followed by extraction into hexane. A sample of known α -tocopherol concentration was run with each batch, as was a straight recovery (water sample with known amount). Recovery was 97 % with a standard deviation of 4 %. Serum samples from day 27 were not submitted for analysis.

Liver samples were taken by biopsy using the method previously described (Loosmore and Allcroft, 1951) and samples were submitted with serum for analysis of α -tocopherol concentration as above.

STATISTICAL ANALYSIS

Analysis of serum and liver α -tocopherol data was performed using analysis of variance and covariance, using the general linear model procedure of SAS (SAS User's Guide, 1988). The full model for the serum data included treatment group, with the concentration of α -tocopherol on day 0 as a covariant. The full model for the liver α -tocopherol data included treatment group, with the concentration of α -tocopherol on day 0 and the weight of the liver samples on the day being tested as covariants. Analysis of the skin score data was performed using analysis of variance ranks, using the general linear model procedure of SAS. The full model for the skin data included treatment group, with the skin scores on day 0 as a covariant. In all of the analyses, treatments are considered as fixed effects. Differences between means were tested using the student t-test. Means reported are least squares means (LSM) \pm SE. Results are considered significant at $P < 0.05$.

RESULTS

The least squares means (LSM) of the serum α -tocopherol data are presented in Table 2.2 and Figures 2.1 and 2.2. Group C was significantly different from control group D on day 2 of the trial, and groups A and C were significantly different from control group D on day 6. After day 6 there was no significant difference between groups.

The least squares means (LSM) of the liver data are presented in Table 2.3. No significant difference was seen between any of the treatment groups for the entire length of the trial. Results from the skin scores are presented in Table 2.4 and show that there is no statistical difference between the skin scores of the control and treatment groups.

Table 2.2. LSM for serum α -tocopherol (mg/l) for cows treated topically with 1 of 4 formulations of vitamin E, and untreated control group (group D). LSM are adjusted for serum α -tocopherol concentration on day 0.

	Group A	Group B	Group C	Group D	Group E	Pooled SE
Day 2	2.18	2.29	2.56 *	2.24	2.24	0.11
Day 6	3.07 *	2.41	2.95 *	2.19	2.63	0.17
Day 14	2.02	1.84	2.03	1.99	1.76	0.11
Day 20	1.99	1.99	2.08	2.13	1.81	0.11

* = Statistically different from respective value for control group D (P<0.05).

Table 2.3. LSM for hepatic α -tocopherol concentration (mg/kg) for cows treated topically with 1 of 4 formulations of vitamin E, and untreated cows (group D). LSM are adjusted for hepatic α -tocopherol on day 0 and weight of liver samples.

	Group A	Group B	Group C	Group D	Group E
Day 6	19.1±3.4	14.3±3.1	19.3±2.7	13.6±3.2	12.6±2.8
Day 20	14.1±4.9	9.9±3.7	7.8±4.3	13.9±3.2	12.1±2.4

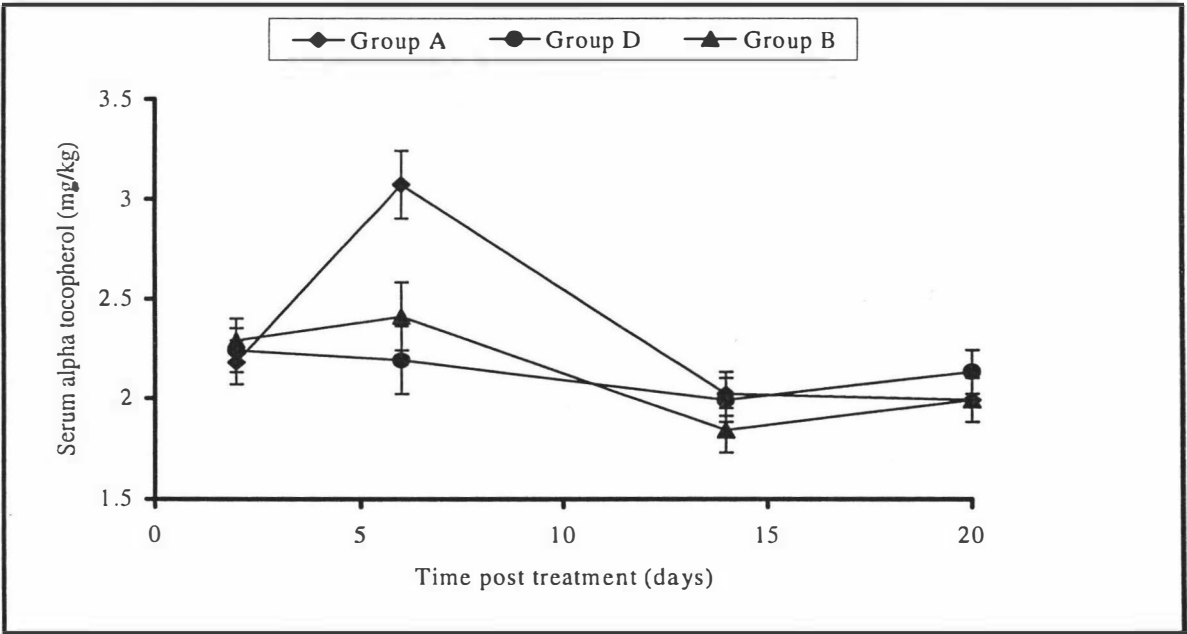


Figure 2.1. LSM of serum α -tocopherol for treatment groups A and B and for untreated control group D plotted against sampling dates. LSM are adjusted for serum α -tocopherol concentration on day 0.

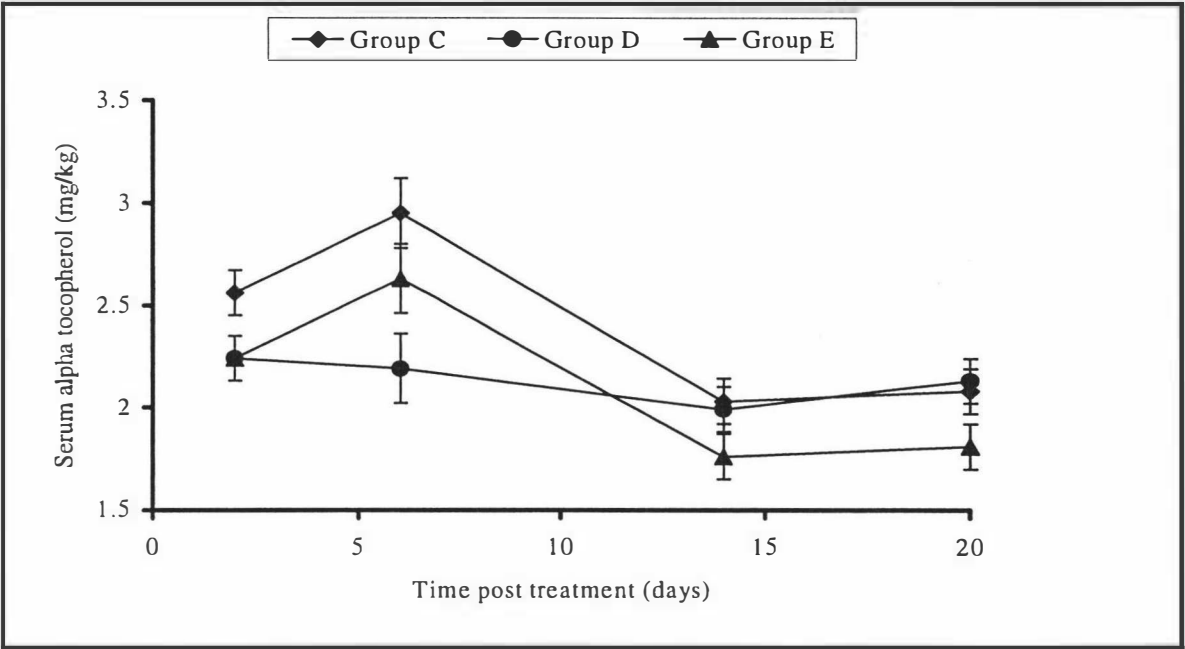


Figure 2.2. LSM for serum α -tocopherol for treatment groups C and E and untreated control group D plotted against sampling dates. LSM are adjusted for serum α -tocopherol concentration on day 0.

Table 2.4. LSM of skin scores for treatment groups. LSM for days 2 to 27 are adjusted for skin scores on day 0.

Group	D0	D2	D6	D14	D20	D27
A	5.0±0	5.0±0	5.0±0	4.33±0.33	4.16±0.48	4.0±0.63
B	4.83±0.16	5.0±0	4.83±0.16	4.83±0.16	4.83±0.16	4.83±0.16
C	4.83±0.16	5.0±0	4.83±0.16	4.5±0.34	4.5±0.34	4.66±0.33
D	4.83±0.16	4.66±0.33	4.83±0.16	5.0±0	4.83±0.16	4.83±0.16
E	4.66±0.21	4.66±0.21	4.83±0.16	4.83±0.16	4.66±0.21	4.66±0.21

DISCUSSION

While the serum concentrations of α -tocopherol for treatment groups A and C are significantly different from controls, the practical importance of these differences may at first appear questionable. The serum levels of these two groups are barely above the threshold for adequacy (2 mg/l) (Rammell and Cunliffe, 1983), and they fall back to levels similar to those of the control group some time between day 6 and 14 of the trial. When these results are compared to experiments performed by other authors using a similar dose of dl- α -tocopherol acetate there are some important practical implications. Charmley *et al* (1992) treated a group of non-lactating dairy cows with 4500 IU of dl- α -tocopherol acetate and found a serum α -tocopherol response very similar to that shown in the current study. The dose rate used in this study was almost twice that of Charmley *et al* (1992) (7700 IU on average). For a pour-on application to give a response similar to that of an injectable when used at only twice the dose rate is very promising. A number of authors have performed experiments showing increased α -tocopherol serum concentrations in cows dosed with α -tocopherol orally, at dose rates much higher than the ones used in this trial (Charmley *et al.*, 1992; Hidioglou *et al.*, 1988), with serum α -tocopherol responses not proportionally as high as the dose rate. It is possible that pour-on A would result in serum α -tocopherol responses equivalent to or greater than those shown by authors who have used the oral route, if pour-on A was given at the same frequency and dose rate. It should be noted that on average, only about 30% of dietary vitamin E seems to be absorbed (Reddy, 1985).

In order for this pour-on formulation to be more effective it would be desirable to increase the serum and liver α -tocopherol response and increase the longevity of its action. The easiest way to do this would be to increase the dose rate or the frequency of doses. Either way would increase the cost of the treatment, therefore it may be more desirable to increase the proportion of α -tocopherol that partitions from the pour-on into the systemic circulation. Changing the type of enhancers used in the pour-on formulations would be the most effective way of doing this. As can be seen in this trial there is a definite difference in the effectiveness of the pour-on formulations used. All of these formulations contain the same amount of vitamin E, therefore the

apparently different absorption rates observed must be due to the different types and concentrations of enhancers used. The enhancers used in this trial were chosen because other authors had shown their ability to promote absorption of lipophilic substances. Claudi (1995) showed that isopropyl myristate (IPM) promoted absorption of a model lipophilic drug (methyl nicotinate) when compared to a non-treated control. Nishihata (1990) found that octanol increased absorption of the model lipophilic drug indomethacin when compared to the absorption of indomethacin alone. A number of other authors (Hori, 1992; Sasaki, 1990) have stated that some enhancers when used in combination increase absorption by a factor that is greater than if they were used alone. These reports lead to the formulation of the pour-ons used in this trial. Formulation 1 contained the enhancer IPM, formulation 2 contained the enhancer octanol and formulation 3 was made up of a combination of these two enhancers. Formulations 1, 2, and 4 all had sesame oil as a base. One of the factors limiting percutaneous absorption is the partitioning of a drug from the formulation to the skin (Riviere and Spoo, 1995). As sesame oil is a lipophilic substance and the penetrant (α -tocopherol) is also lipophilic there was some concern that the penetrant may have a greater affinity for the formulation than the skin and hence not partition from the formulation into the skin. To test if this occurred, formulation 3 was used. This formulation did not have a lipophilic base so it was hypothesised that the penetrant would partition from the vehicle into the skin more easily, resulting in a higher penetration rate. This was not the case since formulation 3 did not produce significantly different serum or liver α -tocopherol concentrations when compared to the control. Formulation 3 did not contain a known penetration enhancer. It is possible that the penetrant was able to pass from the pour-on to the skin surface but it did not have sufficient activity to pass through the skin. The two formulations that gave significant improvement in absorption over the control both contained sesame oil. The presence of the enhancers in the formulation not only promoted absorption through the skin but probably also enhanced partitioning from the original formulation.

A number of other authors have shown that there are a variety of enhancers available to promote absorption of lipophilic substances. Different alkanols have been shown to increase absorption of lipophilic substances by Nishihata (1990) and Hori (1992).

Testing of some of these enhancers in future trials may result in the identification of other enhancers that promote higher absorption rates.

Liver α -tocopherol concentrations were not different for any of the groups at any point in this trial. Standard errors were large indicating an inherent variability in hepatic α -tocopherol concentration. To overcome this problem, a future trial could be performed using a larger number of liver biopsies. Increasing sample size is the easiest way to overcome variability problems. While liver is a storage organ for vitamin E, it only accumulates a few day's supply (Puls, 1994), and the kinetics of α -tocopherol in hepatic tissue is likely to reflect that observed in serum.

It can be concluded that topically applied α -tocopherol can enter the systemic circulation and increase serum α -tocopherol levels significantly. It is also suggested by this author that α -tocopherol supplementation using pour-on A, would result in blood responses that are equivalent to those of an oral dose of this vitamin given at the same dose rate. This needs to be tested using further clinical trials. Although bioequivalence with currently registered vitamin E supplementation products may be achieved using pour-on A, modifying the formulation could increase the longevity of action of the pour-on resulting in a superior product that may have a more promising commercial application.

**CHAPTER 3: SERUM AND LIVER
CONCENTRATIONS OF VITAMIN B₁₂
AFTER THE APPLICATION OF TWO
VITAMIN B₁₂ POUR-ON FORMULATIONS**

ABSTRACT

Thirty Hereford yearling cattle were used to investigate the concentration of vitamin B₁₂ in plasma and liver following the topical administration of 2 vitamin B₁₂ pour-on treatments. Animals were allotted to the following groups: B₁₂ pour-on A; B₁₂ pour-on B; and a control group. Animals in treatment groups received a dose of 6 mg cyanocobalamin per 50 kg live weight of their allotted pour-on on the first and seventh days of the trial. Blood samples were collected from all cows on days 0, 2, 7, 9, and 14 for assay of plasma vitamin B₁₂ concentration. Liver samples were collected from 5 of the cows in each group on days 0, 7, and 14 and were also analysed for vitamin B₁₂ concentration.

Differences between treatment groups in plasma and liver vitamin B₁₂ concentrations were not significant. It was concluded that cyanocobalamin did not cross the skin in sufficient amounts to elicit a blood or liver response.

INTRODUCTION

Vitamin B₁₂ functions as a coenzyme in transmethylation reactions, and deficiency impairs DNA synthesis. Vitamin B₁₂ is also required for tricarboxylic acid cycle function. This is particularly important in ruminants because propionate, a major end product of ruminal fermentation of carbohydrates, can not enter the tricarboxylic acid cycle or be used for gluconeogenesis in the absence of vitamin B₁₂ (Naylor and Ralston, 1991). The effects of vitamin B₁₂ deficiency on the energy metabolism of ruminants can explain the signs of poor growth and ketosis seen in vitamin B₁₂ deficient animals.

To overcome deficiency, ruminants are supplemented with vitamin B₁₂ or cobalt. To date ruminants have been supplemented by means of a vitamin B₁₂ injection or oral supplementation in the form of cobalt sulphate. Cobalt is used by the rumen micro-organisms for the synthesis of vitamin B₁₂. Previous authors have shown that vitamin B₁₂ or cobalt supplementation has resulted in bodyweight gains in cobalt deficient animals (Judson *et al.*, 1982; Skerman *et al.*, 1959).

The purpose of this trial was to examine the effect of treatment with pour-on formulations containing vitamin B₁₂ on plasma and liver vitamin B₁₂ concentrations in cattle.

METHODS AND MATERIALS

Thirty male Hereford yearling cattle with an average weight of 284 kg were randomly allocated to 1 of 3 groups (A, B and C) of 10 animals, balanced for liveweight. The animals used in this trial were selected because they had previously been shown to have low baseline values of vitamin B₁₂. Two of the 3 groups (A and B) were assigned a pour-on treatment and the remaining group (C) was assigned no treatment and acted as the control. The formulations of the 2 pour-on treatments are shown in Table 3.1. All of the animals in groups A and B were treated with a vitamin B₁₂ pour-on at a rate

of 6 mg of cyanocobalamin per 50 kg liveweight on the first and seventh days of the trial. The pour-on formulation was applied along the midline of the back between the shoulder and tail head using a graduated syringe. Blood samples were taken from all animals on days 0, 2, 7, 9, and 14. Samples taken on day 0 and 7 were obtained before the treatments were applied. Blood was drawn from the jugular vein into plain vacutainers and stored overnight before being submitted to the Lincoln Animal Health Laboratory, Ministry of Agriculture for analysis. Liver samples were taken by aspiration biopsy as described by Loosmore and Allcroft (1951), from 5 animals in each of the trial groups on days 0, 7 and 14. Liver samples were stored in vacutainers and submitted with the blood samples. All of the animals in the trial remained in a paddock that was directly adjacent to the yards for the entire length of the trial, except on the days that they were sampled. On these days the animals were brought into the yards for approximately 1 to 2 hours with the exception of day 0. On this day a group of 60 animals were yarded for approximately 3 hours. The animals used in this trial were then drafted off from this group.

A brief description of the vitamin B₁₂ analysis procedures follows. Vitamin B₁₂ in the sample and control was extracted from its protein complex by boiling at pH 4.0. A known amount of radioactively labelled vitamin B₁₂ was then allowed to compete with the extracted vitamin B₁₂ for binding to a limited amount of vitamin B₁₂ binding protein (derived from chick sera). Bound and free portions of vitamin B₁₂ were separated by adding a charcoal/albumin slurry, then centrifuged and decanted. Measurement of the amount of labelled vitamin B₁₂ that is bound allows determination of the degree of radioisotopic dilution (using LKB Wizard gamma counter software), and subsequently the amount of vitamin B₁₂ present in the sample by comparison to a standard curve (Newmark *et al*, 1974). For daily quality control, three levels of Biorad Lyphocheck commercial control specimens were used. The laboratory is Telarc registered for this test.

Analysis of plasma and liver vitamin B₁₂ data was performed by analysis of variance, using the general linear model procedure of SAS (SAS User's Guide, 1988). In all analyses, treatments are considered fixed effects. Differences between means were tested using the students t-test. Means reported are least squares means (LSM) \pm SE.

Table 3.1 Formulation of pour-on treatments A and B

Formulation	Component	Proportion (%)
A	Cyanocobalamin	0.24
	Ethanol	59.5
	Propylene glycol	40.26
B	Cyanocobalamin	0.24
	Ammonium sulphate	0.5
	Butyl dioxitol	30
	Distilled water	69.26

RESULTS

Vitamin B₁₂ pour-on treatments A and B had no effect on plasma or liver vitamin B₁₂ concentrations when compared to the control group. The LSM of liver and plasma concentrations of the different trial groups are shown in Table 3.2 and Table 3.3 respectively. These results are also presented graphically in Figures 3.1 and 3.2.

Table 3.2. LSM of liver vitamin B₁₂ concentrations (nmol/kg) in yearling cattle treated with vitamin B₁₂ pour-on formulation A or B and an untreated control group.

Trial group	Day 0	Day 7	Day 14
Pour-on A	350	138	164
Pour-on B	294	140	144
Control	274	147	174
Pooled SE	±86.65	±28.96	±23.01

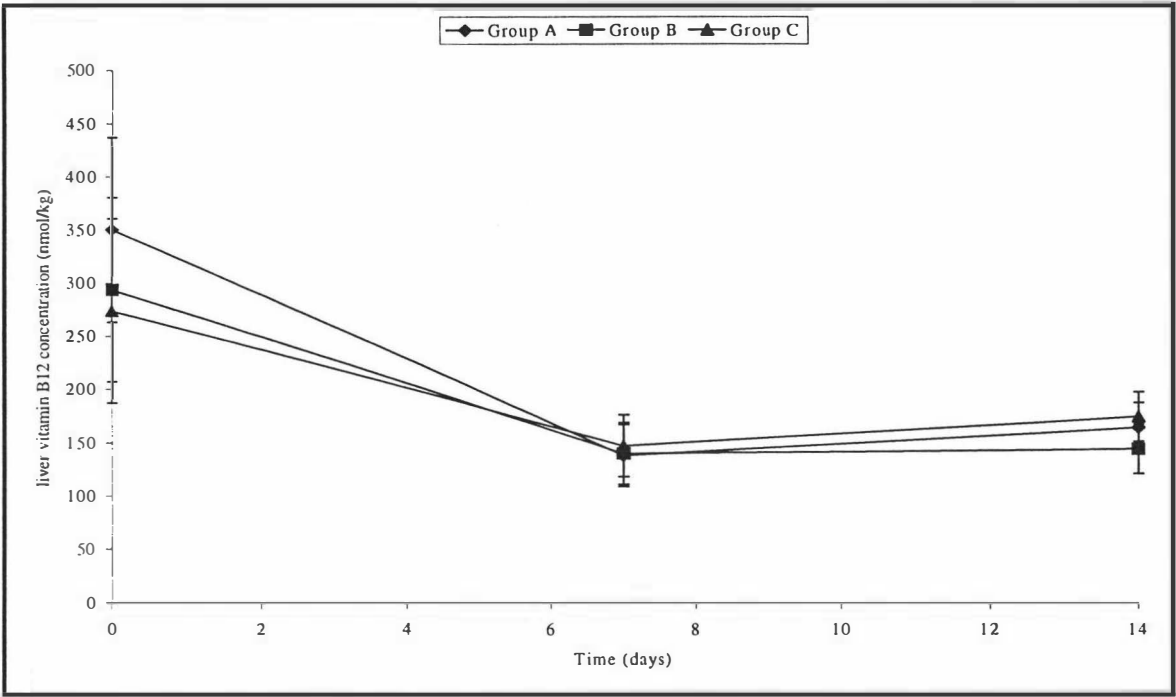


Figure 3.1. LSM of liver vitamin B₁₂ concentration (nmol/kg) for yearling cattle treated with a vitamin B₁₂ formulation (groups A and B) and an untreated control group C.

Table 3.3. LSM of blood plasma vitamin B₁₂ concentration (pmol/l) in yearling cattle treated with vitamin B₁₂ formulation A or B and untreated control group.

Trial group	Day 0	Day 2	Day 7	Day 9	Day 14
Pour-on A	591	72	125	137	148
Pour-on B	668	70	118	104	105
Control	609	74	245	103	116
Pooled SE	±130.72	±8.49	±82.11	±12.55	±15.92

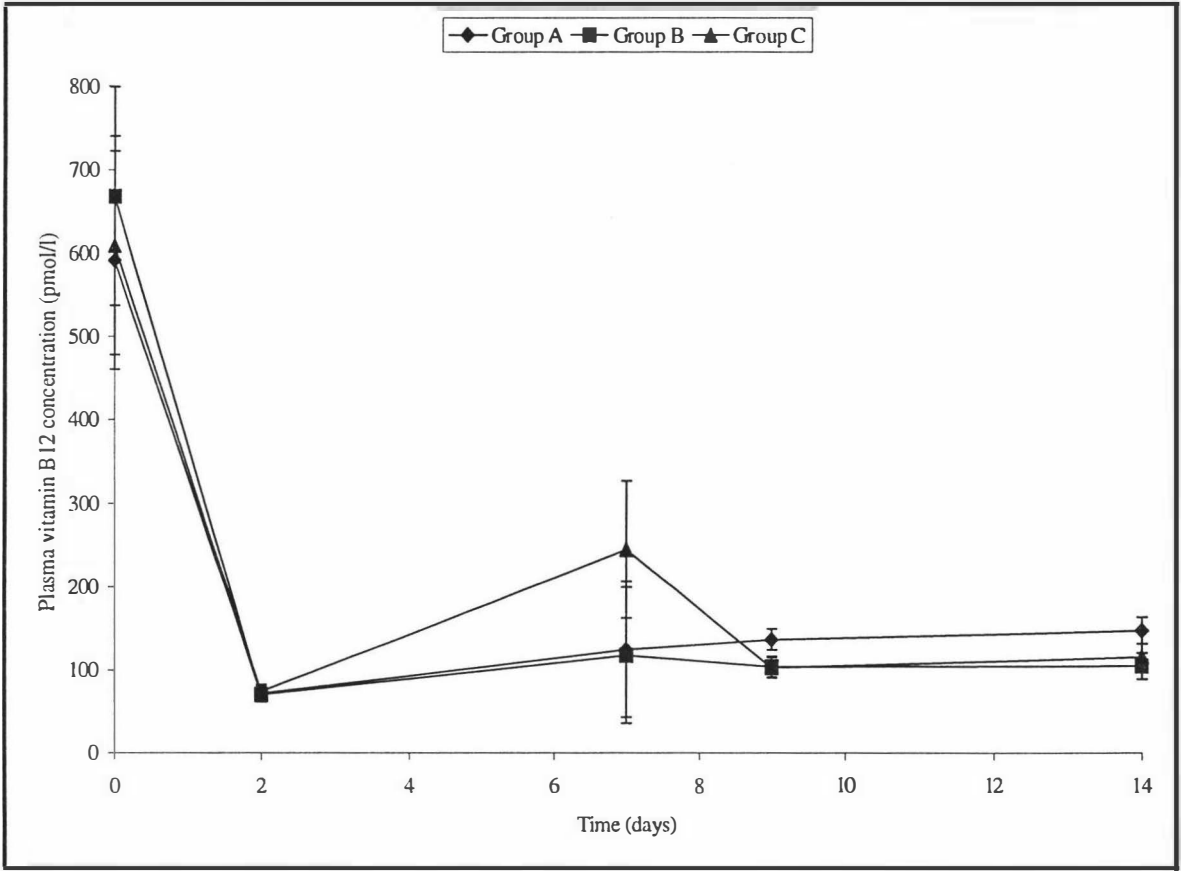


Figure 3.2. LSM of plasma vitamin B₁₂ concentration (pmol/l) in yearling cattle treated with vitamin B₁₂ pour-on formulation A or B and an untreated control group C.

DISCUSSION

Neither blood nor liver vitamin B₁₂ concentrations increased in the treatment groups when compared to the control group. The rain that fell after the application of the pour-ons may have affected this result. In the 48 hrs following the first treatment a rainfall of 89 mm was recorded. Both of the pour-on formulations used in this trial are water-soluble and it is possible that the amount of rain that fell could have washed the treatments off the animals backs before maximal absorption could occur.

Due to the poor weather and the possibility that the pour-on treatments may have been washed off, it was decided that the animals would be treated again on day 7. A second treatment was deemed appropriate, as the object of this preliminary trial was to investigate if vitamin B₁₂ could be absorbed. After the application of the pour-on treatments on the seventh day, there was still no response in liver or blood vitamin B₁₂ levels. Again there was a problem with the weather, with a recorded rainfall of 20 mm in the 48 hr after the application of the day 7 treatment. While this amount of rain would probably not have completely washed off the treatments, it still could have diluted the constituents included in the pour-on formulations that might have enhanced cyanocobalamin transport through the skin. This is particularly important in the case of pour-on A, which contained ethanol at a concentration of 75 %. Hatanaka (1994) showed that ethanol enhances skin permeability of hydrophilic compounds at high concentrations, whereas at low concentrations it enhances skin permeability of lipophilic substances. Vitamin B₁₂ is a hydrophilic compound, therefore the ability of the ethanol in pour-on A to enhance the penetration of vitamin B₁₂ across the skin could have been reduced by dilution of the ethanol in the pour-on formulation due to the rain.

The concentration of vitamin B₁₂ in the formulations may not have been high enough to elicit a blood or liver response. Clark *et al* (1986) observed an increase in blood concentrations of vitamin B₁₂ in a group of cattle treated with 2 doses of 2 mg per head of hydroxocobalamin by intramuscular injection when compared to a control group. These doses were given at an interval of one month and the response observed

was significant one month after the second dose. Hydroxocobalamin is a less soluble form of vitamin B₁₂ than the form that was used in this trial. Peak blood levels of hydroxocobalamin are achieved 72 hours after injection whereas cyanocobalamin raises blood levels of vitamin B₁₂ almost immediately after injection (Ethical Agents Ltd). The frequency of sampling and the size of the dose that was applied (12 times as much as Clark administered) in the present study should have allowed detection of increased vitamin B₁₂ in liver and serum if the vitamin was absorbed.

It can be concluded from this trial that the vitamin B₁₂ in the pour-on formulations tested in this study did not cross the skin at high enough levels to elicit an increase in blood or liver concentrations. While the results may have been affected by the weather, it is possible that the formulations used are not ideal. To test if this is the case, the formulations used in this trial could be retested in an *in-vitro* permeation model, or the experiment repeated in an environment where weather effects are not significant.

**CHAPTER 4: *IN-VITRO* AND *IN-VIVO*
EXAMINATION OF THREE SELENIUM
POUR-ON FORMULATIONS**

ABSTRACT

The dermal permeability of 3 selenium pour-on formulations was examined over 24 hours using an *in-vitro* calf skin permeation model. Pour-on formulation A had a statistically greater rate of absorption than formulations B or C for the 24 hour period. The absorption rates of formulations B and C were not statistically different. The results obtained from the *in-vitro* experiment were compared with those from an *in-vivo* experiment using the same pour-on formulations, each applied to 6 cattle. Comparison of the 2 data sets indicated that the *in-vitro* model was useful in differentiating the formulations order of *in-vivo* permeability.

INTRODUCTION

Pitman and Rostas (1982) suggested that screening tests for cattle and sheep skin permeability which employ skin sections from dead cattle or sheep have the potential to provide useful information to developers of veterinary topical dosage formulations. Using an *in-vitro* model as a screening test for pour-on formulations which are in the development stage, would be beneficial to pour-on research as a model is less costly, easier to monitor and does not have any of the welfare implications associated with live animal testing. In order for a model to be of use, not only must it show differences in permeability between different pour-on formulations when they exist, but the results must be comparable to those shown in live animals. While previous authors have performed experiments using cattle hide in permeation experiments (Pitman and Rostas, 1982/3; Yazdanian and Chen, 1995), none of these authors have correlated this data with *in-vivo* results. It is the objective of this trial to develop a model that can be used to rank formulations on their ability to enhance the permeation of selenium through cattle skin and show that these results are comparable to those found in the live animal.

METHODS AND MATERIALS

IN-VITRO

Preparation of skin

Skin samples taken from 1 stillborn Friesian dairy calf were used for all of the experiments performed in this study. The hair was clipped as close as possible to the skin shortly after the animal was born, and skin samples were excised from the dorsal thoracolumbar region. The skin samples were stored in air-tight plastic bags at -20°C. Under these storage conditions, the permeation properties of bovine skin do not change for up to 6 months (Yazdanian and Chen, 1994). Before mounting in the diffusion cell, the skin samples were thawed at room temperature and any loose connective tissue was removed by dissection with a scalpel blade.

Diffusion cell setup

The design of the diffusion cell used in this study was based loosely on that of the Franz diffusion cell (Franz, 1975). Each cell consisted of a lower glass chamber, with a side arm to allow sampling of the receptor phase and an upper glass chamber through which the treatment was applied. Both of these chambers were clamped around a piece of calf skin to form the final diffusion cell (see Figure 4.1). The receptor chamber was filled with saline solution, which also contained 0.02 % sodium azide to discourage autolysis of the skin.

As each cell was assembled it was placed in a water bath so that the water level was just below the lip of the lower chamber. The skin in the diffusion cells was never in contact with the water in the water bath. The temperature of the water bath was maintained at $37 \pm 1^{\circ}\text{C}$. Before experiments were started the cells were allowed to equilibrate for 15 minutes. Once equilibrated, each cell was removed from the water bath and a 6 ml sample was extracted from the receptor compartment. This sample was replaced by fresh saline solution, which was also kept at 37°C . Once the first sample was taken, each cell was treated with the appropriate pour-on formulation. The pour-on was applied to the epidermal side of the skin through the upper chamber. This chamber was then closed off with a piece of paraffin film. Samples were then taken by the same method as above at 3, 6, 12, 18 and 24 hours post-treatment. At the conclusion of the experiment, the skin samples were removed from the diffusion cells and frozen overnight so that their thickness could be measured the next day. The average of 6 readings taken randomly over the entire permeation surface was recorded. The entire experiment was repeated six times.

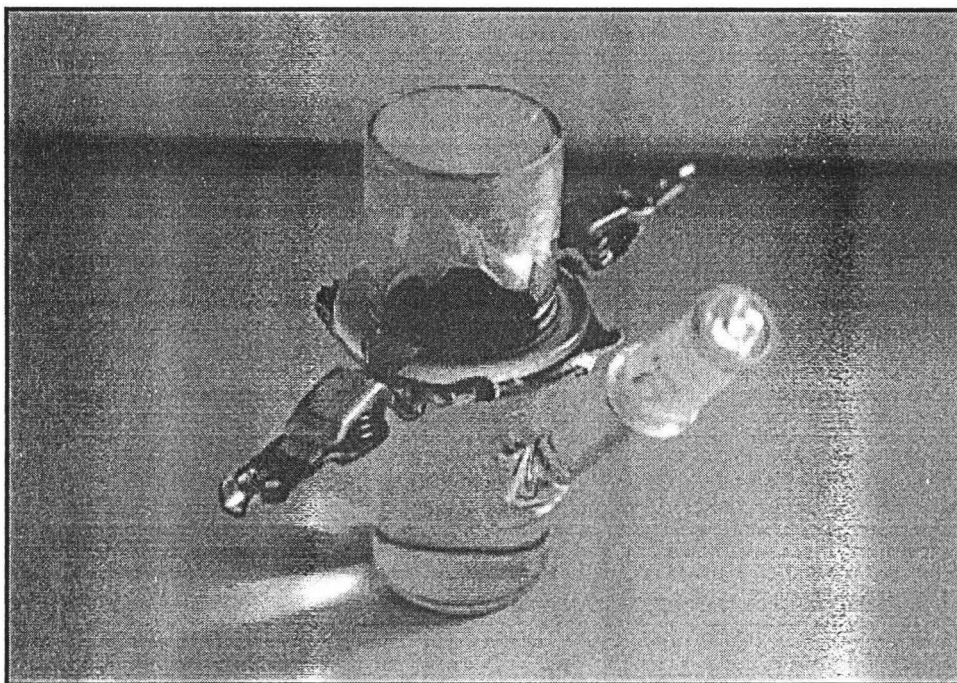


Figure 4.1. Diffusion cell developed to examine the diffusion of 3 selenium pour-on formulations through cattle skin.

Maintenance of skin barrier properties.

In order to show that skin barrier properties were maintained an experiment was performed to examine the permeation of albumin through calf skin. It was assumed that albumin would not cross the skin if its barrier properties were intact due to the size of this molecule. All cells used in this experiment were set up as above. Four cells were treated with 5 ml of a saline bovine serum albumin solution at a concentration of 50 grams albumin / litre saline. Four more cells were treated with 5 ml of saline and acted as the control. Samples were taken as described above at 0, 3, 6, 12 and 24 hours and submitted to the Massey University clinical pathology laboratory for albumin analysis.

***IN-VIVO* STUDY.**

Twenty-four Friesian dairy cows were randomly allocated to 4 groups of 6 animals labeled A, B, C and D. Selenium pour-on treatments A, B and C were used to treat groups A, B and C respectively and group D received no treatment and acted as the control group. The formulations of the selenium pour-on treatments used in this trial

are shown in Table 4.1. Blood samples were obtained from all animals prior to treatment on day 0 and again at 3 hours, 6 hours, 12 hours, 18 hours, 24 hours, 48 hours, 7 days, 14 days, 21 days, 28 days, 42 days, and 58 days post-treatment. Treatments were applied along the midline of the back between the shoulder and tail head using a graduated syringe to give a dose of 7.5 mg of selenium per 50 kg bodyweight.

Sample collection and method of analysis

Blood samples were obtained via caudal venipuncture and were collected into plain evacuated plastic tubes. Tubes were kept under refrigeration for 24 hours then centrifuged and the serum decanted. Serum samples were then submitted to the Ruakura Animal Health Laboratory, Ministry of Agriculture, for selenium analysis. The concentration of selenium in serum was measured by the fluorometric method (Watkinson, 1979, 1977; Watkinson and Brown, 1979). Values for each batch were standardised using a control included in each daily batch. A commercially freeze-dried control (660 nmol/l) was also included as an external control.

ANALYSIS OF DATA

Analysis of selenium data and albumin data was performed by analysis of variance and covariance, using the general linear model procedure of SAS (SAS User's Guide, 1988). The full model for the serum data included treatment group, with the concentration of selenium at time 0 as a covariant. The full model for the saline selenium and albumin data included treatment group only. Associations between treatment variables were tested using the students t-test. Means reported are LSM \pm SE. Results were considered significant at $P < 0.05$.

Table 4.1. Formulations used for the *in-vivo* and *in-vitro* selenium pour-on trials.

Pour-on	Formulation	%W/V
A	Selenium Dioxide	0.71
	Butyl Dioxitol	99.29
B	Sodium Selenate	1.2
	Butyl Dioxitol	30
	Water	68.8
C	Selenium Sesquistrato	3.05
	Butyl Dioxitol	30
	Water	66.95

RESULTS

IN-VITRO

Concentration of selenium at time 0 was analyzed as a covariant and had no effect on treatment so was excluded. Group A showed statistically significant greater concentrations of selenium than groups B and C for all of the sample times. Groups B and C were not significantly different from each other for the entire length of the trial. Least squares means (LSM) of the concentrations of selenium in saline are presented in Table 4.2 and Figure 4.2. Skin thickness was examined as a covariant and shown to have no significant effect on absorption.

IN-VIVO

Least squares means of serum selenium concentration, adjusted for day 0, are presented in Table 4.3 and Figures 4.3 and 4.4. Serum selenium concentrations of cows that were not supplemented with a selenium pour-on remained in a range

between 200 and 300 nmol/L throughout the study. Serum selenium concentrations of the cows supplemented with pour-on A increased to a mean of 990 nmol/l at 3 hours post treatment then proceeded to fall to a mean of 329 nmol/l on the final sample date (day 58). Serum selenium concentrations were significantly different from the control group for the entire length of the trial excluding day 1. Both groups B and C did not reach levels above those of the control group until 18 hours post treatment. They then remained significantly different from the control group for the entire length of the trial with the exception of group B at day 14 and group C at day 58. Groups B and C were not statistically different from each other throughout the trial.

COMPARISION

The LSMs of the whole of the *in-vitro* trial and the first 24 hours of the *in-vivo* trial are presented in Figures 4.2 and 4.3 respectively. Treatment group C had an absorption profile that is very similar *in-vitro* and *in-vivo*. Both *in-vitro* and *in-vivo* results show a steady increase in selenium concentration between 0 and 24 hours. The *in-vitro* absorption profile of treatment group B shows a steady increase in absorption between 0 and 18 hours with selenium concentration dropping by 24 hours. In the *in-vivo* study, the steady increase is only observed up to the 12 hour mark with a decrease occurring from 12 to 24 hours. Treatment group A had an *in-vitro* absorption profile that shows a steady increase in selenium concentration from 0 to 12 hours and a decline in concentration from 12 to 24 hours. The *in-vivo* profile shows a rapid rise in concentration by 3 hours and a decline in concentration from this point on.

BARRIER PROPERTIES

The LSM from the albumin experiment are presented in Table 4.4. The treatment group and control group are not significantly different from each other at any of the sampling times.

Table 4.2. LSM of saline selenium for *in-vitro* cells treated with 1 of 3 selenium pour-on formulations.

Time (hrs)	Saline selenium concentration (nmol/l)			Pooled SE
	Group A	Group B	Group C	
0	9	57	30	±24
3	93245	14460	4395	±18746
6	236650	44917	27300	±41336
12	420000	106000	72833	±30922
18	430000	129667	91000	±29686
24	363333	122667	110333	±23541

Table 4.3. LSM of serum selenium concentration for cows treated topically with 1 of 3 selenium pour-on formulations, and an untreated control group D. The LSM are adjusted for serum selenium concentration of day 0.

Time	Serum selenium concentration (nmol/l)			
	Group A	Group B	Group C	Group D
3 hrs	990.75±89.96	296.65±88.99	300.95±88.31	248.33±88.26
6 hrs	903.19±77.06	357.80±76.24	339.12±75.65	263.23±75.61
12 hrs	725.83±61.19	424.93±60.53	359.26±60.07	274.99±60.04
18 hrs	652.40±35.43	384.96±35.05	386.99±34.78	267.32±34.76
24 hrs	659.96±34.06	388.27±33.96	401.46±33.44	256.99±33.42
48 hrs	744.12±35.49	556.61±35.10	597.60±34.84	269.99±34.82
7 days	712.96±70.84	534.05±70.08	537.84±69.54	258.48±69.51
14 days	500.29±48.64	441.98±48.12	446.67±47.75	306.06±47.43
21 days	543.41±30.45	422.62±30.13	393.44±29.90	295.52±29.88
28 days	364.01±17.36	297.25±17.18	292.28±17.05	199.78±17.04
42 days	374.45±20.36	353.47±20.14	332.25±19.99	254.16±19.97
58 days	329.15±16.50	338.26±16.33	295.93±16.20	269.99±16.19

Table 4.4. LSM of albumin concentration for *in-vitro* cells treated with albumin solution (50 g/l) and an untreated control group.

Time (hrs)	Saline albumen concentration g/l	
	Treatment Group	Control Group
0	0.09 ± 0.063	0.003 ± 0.063
3	0.065 ± 0.014	0.067 ± 0.014
6	0.100 ± 0.019	0.103 ± 0.019
12	0.150 ± 0.019	0.163 ± 0.019
24	0.213 ± 0.020	0.237 ± 0.020

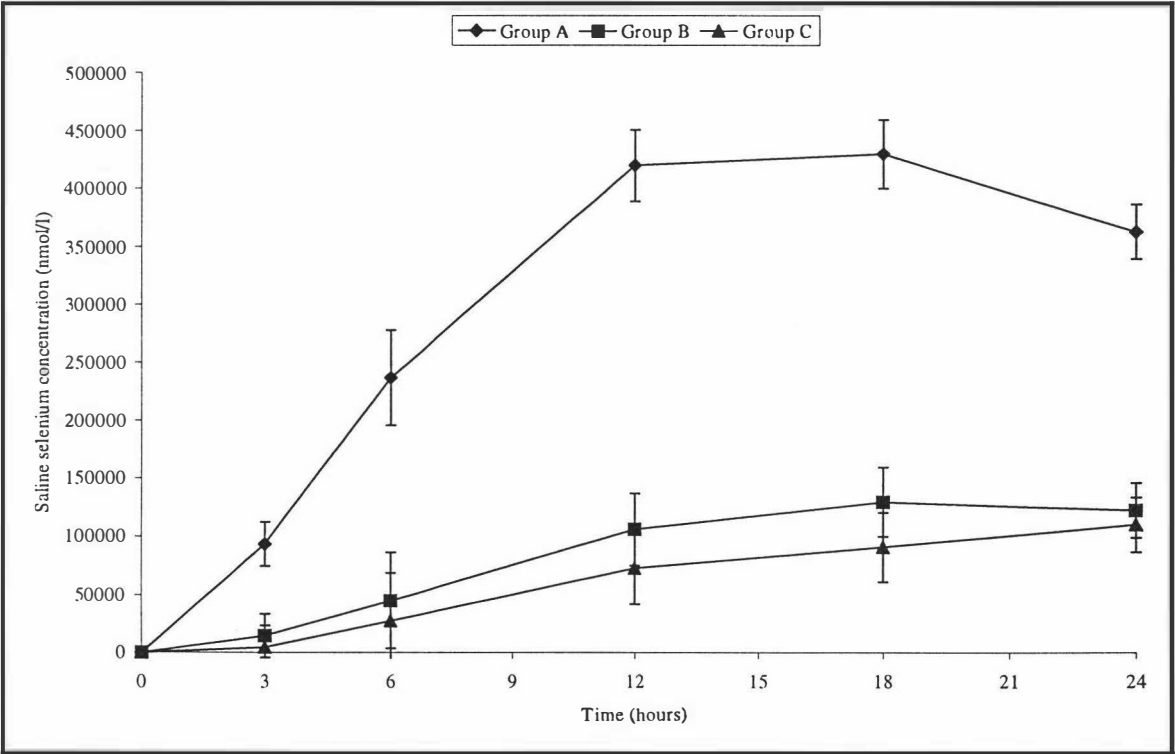


Figure 4.2. LSM of selenium concentration in saline for *in-vitro* cells treated with 1 of 3 selenium pour-on formulations.

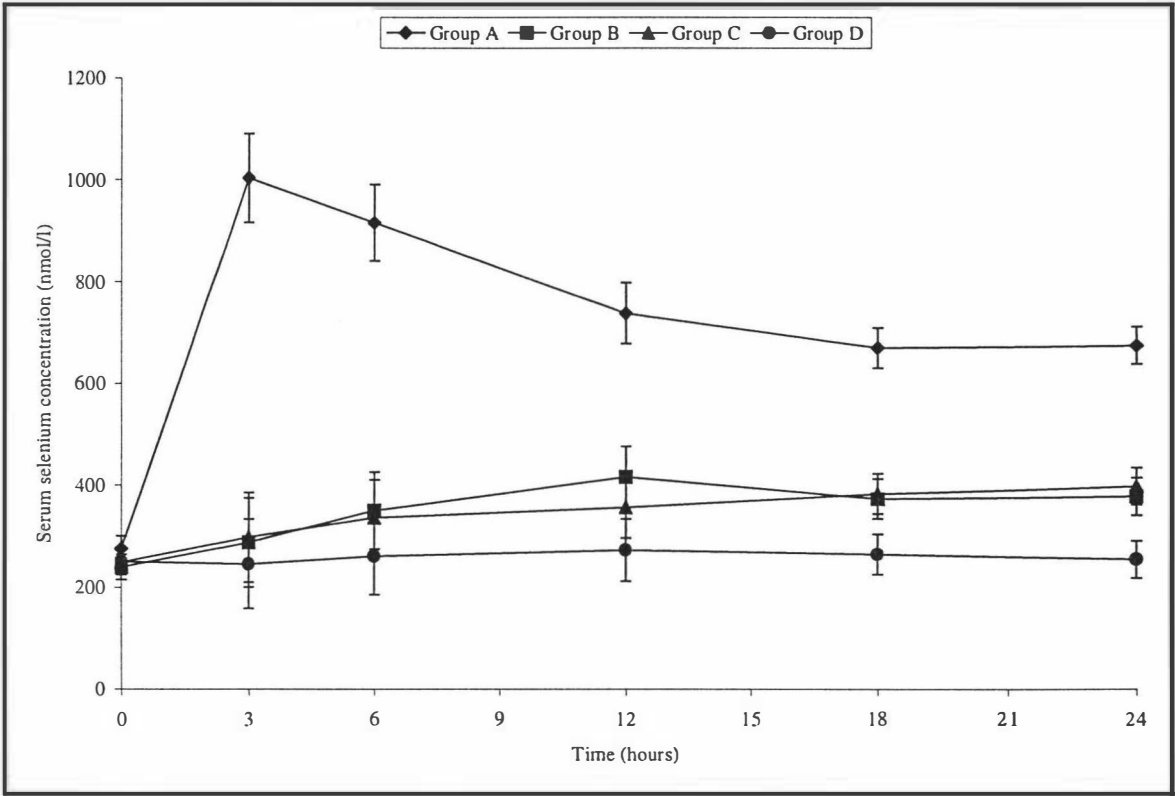


Figure 4.3. LSM of serum selenium concentration for cows treated topically with 1 of 3 formulations of selenium, and an untreated control group D, for the first 24 hours.

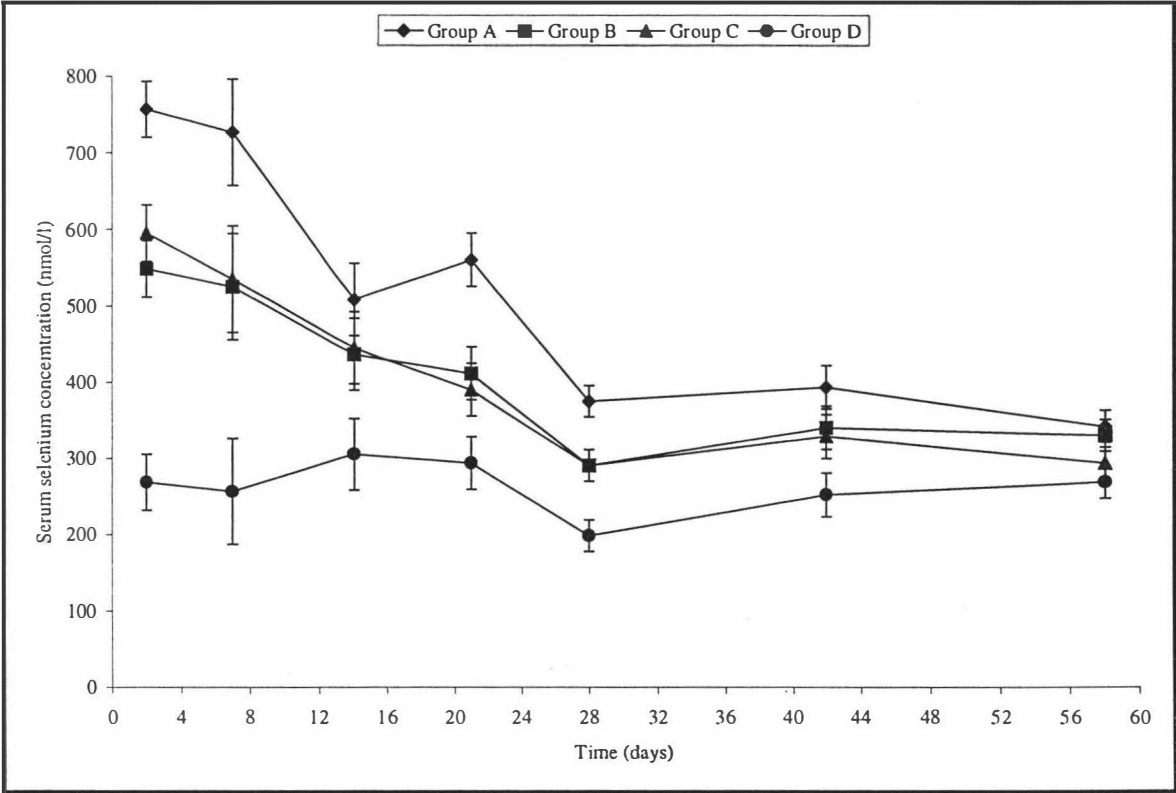


Figure 4.2. LSM of serum selenium concentration for cows treated topically with 1 of 3 selenium pour-on formulations, and an untreated control group D, for days 1 to 60.

DISCUSSION

This study demonstrates that the *in-vitro* excised calf skin technique is capable of differentiating compounds of low permeability from those of high permeability, and ranking them in an order which is equivalent to that seen *in-vivo*. Absorption of all the formulations appears to occur at a faster rate *in-vivo* than *in-vitro*. This may be because the skin is exposed to a proportionally greater dose of selenium per surface area *in-vitro*, resulting in a constant rate of absorption or a steady state being observed over the first 12 hours of the experiment. In the *in-vivo* experiment the skin is exposed to a proportionately small dose of selenium per surface area, therefore no steady state or a short steady state is shown giving the impression that absorption is occurring at a faster rate. The reason that a steady state is not maintained in the *in-vitro* experiment is due to the fact that fresh saline is being added to the receptor chamber after each sample has been taken. This results in a dilution of the selenium concentration in the receptor chamber after maximum absorption has occurred. To test if absorption occurs faster *in-vivo* an experiment would need to be set up that exposed the skin of both the *in-vitro* and *in-vivo* recipients to an infinite amount of selenium over a specified period of time. The slope of the resulting curves could then be examined to see which rate of absorption was greater.

Rapid penetration of drugs formulated as pour-on treatments is desirable. Exposure to the environment will tend to remove or degrade the product soon after its application and it would be unlikely that the product would remain in contact with the skin for a prolonged period of time (Ponting, 1979). Pour-on A had produced a peak selenium concentration by 3 hours after application, with pour-ons B and C both reaching their peak level by 48 hours. The rapid penetration shown by pour-on A suggests that it would be the most effective selenium pour-on formulation tested in this trial.

A number of other authors have performed experiments in the past using excised cattle hide for *in-vitro* permeation experiments (Pitman and Rostas, 1982/3; Yazdanian and Chen, 1995). In all of these experiments the skin used was trimmed to a specified thickness before the commencement of experiments, resulting in skin sections that had

much of the dermis removed. Compounds that are absorbed through the skin *in-vivo* are taken up by blood vessels directly beneath the epidermis, so they are not required to penetrate the full thickness of the skin. It can therefore be assumed that the thick dermal tissue may present a barrier to penetration in the model, which does not exist *in-vivo* (Bronaugh, 1989). This being the case, there was some concern that the penetration profiles of the pour-on formulations used in this trial may be highly variable due to the fact that full thickness skin was used. This may have lead to results that showed little between formulation variation. This was not the case. While there was variability within groups, it did not affect the ability to demonstrate contrasting permeation rates between treatment groups.

Although each of the formulations used in this study contained a different form of selenium, the difference in absorption may be related to the amount of butyl dioxitol in the formulation. Formulations B and C contain the same amount of butyl dioxitol and show very similar absorption rates. Formulation A contains over 3 times as much butyl dioxitol as formulations B and C and shows an absorption rate that is far greater than that shown by formulations B and C. Yazdanian (1995) found that ivermectin permeation through bovine skin was enhanced by another penetration enhancer (DGME) and this enhancement was concentration-dependent. Further studies using only 1 form of selenium and a range of butyl dioxitol concentrations would need to be performed to prove this.

It can be concluded from this experiment that the *in-vitro* model which was used, can differentiate formulations with differing permeability's and rank them in an order that is equivalent to that seen *in-vivo* for the 3 selenium pour-on formulations tested. Using this model as a screening test for pour-on formulations in the future may greatly reduce the need for live animal experimentation.

GENERAL DISCUSSION

The formulations for many licensed pour-on preparations have remained unpublished for reasons of commercial sensitivity, resulting in little information being available on the research and design of products applied by pouring onto the skin. The objective of this thesis was to review the formulation of topically applied pharmaceuticals for both human and animal application and devise ways of producing and testing novel pour-on treatment formulations for cattle.

Two trials were conducted to examine the permeation of vitamin B₁₂ and vitamin E through cattle skin, neither of which had been examined before. A third trial was carried out to examine the permeation of 3 selenium pour-on formulations *in-vivo*. The results from this trial were compared with *in-vitro* experiments that were performed using a model designed specifically for this purpose. Selenium has been shown to cross cattle skin in quantities large enough to elicit a blood response and Ancare NZ Ltd currently has on the market a selenium product applied as a pour-on formulation.

The vitamin E in 2 of the pour-on formulations tested crossed cattle skin at high enough levels to increase serum α -tocopherol concentration. This response was not unexpected, as Sobel and Rosenberg (1953) demonstrated the absorption of topically applied vitamin A, another fat soluble vitamin in rats. The absorption of vitamin D in rats has also been demonstrated by Schaefer *et al* (1956).

Vitamin B₁₂ did not cross the skin in sufficient amounts to alter serum vitamin B₁₂ concentrations. This was unexpected as it has been shown by Howe *et al.* (1967) that vitamin B₁₂ in an ethanol vehicle will cross rat and guinea pig skin at levels high enough to evoke a growth response. Increases in concentration of vitamin B₁₂ in urine and body tissues was also shown by these authors. They found that a large proportion of the applied vitamin B₁₂ was accumulated in the skin, forming a depot from which the vitamin was slowly released into the systemic circulation. It is possible that some of the vitamin B₁₂ in the pour-on formulations used in the trials reported here did partition into the skin but not into the systemic circulation, or the rate at which the vitamin entered the blood supply was so slow that no detectable response was seen.

Research to date on pour-on formulations has predominantly been carried out by testing new products on live animals. This method of testing is time consuming, expensive and can potentially affect the well-being of the animals concerned. One of the main aims of the research conducted and reported in this thesis was to design a device that could be used to test pour-on formulations in the development stage, thus reducing the need for live animal experimentation. The model developed in this study was able to rank the formulations in the same order of permeability as that observed in live animal experimentation. While other authors have examined the permeation of chemicals through cattle skin *in-vitro* (Pitman and Rostas 1982/3; Yazdanian and Chen 1995), none of these authors have compared their results with *in-vivo* experiments. The results shown in chapter 4 of this thesis bode well for future researchers. While none of the other authors have compared *in-vitro* and *in-vivo* permeability, their *in-vitro* data alone can be compared with ours. None of the authors performing *in-vitro* experiments have examined permeation through full thickness skin as was done in our experiment. Large variation in results between replicates was shown by this author while differences between formulation absorption rates was demonstrated. Large variation between replicates has been shown with the use of sheep skin with the majority of its dermis removed in an *in-vitro* model (Ponting 1979). Like us, Ponting (1979) was still able to demonstrate different absorption rates between formulations, suggesting that whole thickness skin is as effective at examining permeation rates as trimmed skin.

RECOMMENDATIONS FOR FUTURE RESEARCH.

While there are a number of commercial pour-on products available, there is little published information relating to any aspect of these products other than their therapeutic effectiveness. This thesis has examined the topic of dermal absorption of veterinary pharmaceuticals in great detail, but there is still a large amount of research that can be performed on this topic. Some of the experiments performed in this thesis could be expanded further. Repeating the selenium trial *in-vitro* using combinations of enhancers and one selenium source would be useful to test for enhancer effects. If certain enhancers result in superior absorption they could also be examined for their ability to promote absorption of other permeants to see if they have a universal effect. Examining tissue accumulation and excretion rates of selenium *in-vivo* may show if

there is any advantage to a formulation that has a high initial absorption. Examining the permeation of the vitamin E pour-on formulations *in-vitro* would result in some more *in-vitro in-vivo* comparisons. Other more general research that can be performed on dermal absorption of chemicals through cattle skin is discussed below.

***In-vitro* model**

It has been firmly established that a relationship exists between the permeability of chemicals through human skin *in-vitro* and *in-vivo*. This fact has lead to the production of a vast amount of quantitative information about the absorption process resulting in more effective formulation design. The research presented in Chapter 4 of this thesis has demonstrated that a relationship between *in-vitro* and *in-vivo* absorption also exists in cattle skin when examining the absorption of selenium. More work needs to be done to see if other chemicals show the same relationship. Characterising mechanisms of absorption will be made easier if the model performs realistically for many penetrants. The experimental methods already developed to study human skin penetration *in-vitro* could be applied to obtain information about the effects of breed, season, age, nutritional status, and formulation on the transdermal delivery process.

It is likely that the *in-vitro* model could also be used to improve the formulations of many of the commercially available anthelmintic formulations. It could also be used to examine the permeation of other novel pour-on formulations, such as formulations containing vaccines or combinations of antelmintics, trace elements and/or vitamins.

Pathway of penetration

It can be assumed that there are only 2 major pathways of penetration for a chemical passing through cattle skin: either through the intact epidermis, or through the skin appendages. Future experiments to define the predominant pathway may result in more effective formulation design as the enhancers can be targeted at the dominant pathway. Possible ways of determining which pathway of penetration predominates are: 1) compare the absorption of a pour-on formulation through the skin of different breeds of cattle with different densities of hair follicles to see if there is an affect on absorption due to hair follicle density; 2) examine the permeation of a chemical through a hairless and hairy piece of cattle skin from the same animal; and 3)

histological examination of skin specimens after the application of a known penetrant using qualitative fluorescent microscopy methods (Rolland *et al.*, 1988) to ascertain the location of the penetrant as it moves through the skin.

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