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An *in-vitro–in-vivo* model for the transdermal delivery of cholecalciferol for the purposes of rodent management

J. Davies [a], A. Ingham [b,*]

[a] School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK
[b] School of Pharmacy, School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK

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ABSTRACT

The natural selection of anticoagulant resistant rats has resulted in a need for an alternative to anticoagulant rodenticides which differs in both active ingredient and in the method of dosing.

Cholecalciferol toxicity to rodents using the dermal route is demonstrated using a variety of penetration enhancing formulations in two *in-vitro* models and finally *in-vivo*.

A 1 ml dose of 50/50 (v/v) DMSO/ethanol containing 15% (v/v) PEG 200 and 20% (w/v) cholecalciferol was judged as '*sufficiently effective*' in line with the European Union's Biocidal Products Regulation (No. 528/2012) during *in-vivo* studies. This dose was found to cause 100% mortality in a rat population in 64.4 h (±22 h).

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1. Introduction

In mammals, cholecalciferol is photosynthesised in the plasma membrane of the dermis when 7-dehydrocholestrol is exposed to wavelengths of light between 290 and 315 nm (Holick et al., 1995; MacLaughlin et al., 1982). Once produced, cholecalciferol is metabolised in the liver and kidneys to form 1,25-dihydroxyvitamin D (Holick and Garabedian, 2006; Holick, 1989, 2009) which is used in calcium homeostasis (Holick, 1989).

Due to their small size, mammals such as rats, mice and rabbits are found to have relatively low tolerance to doses of cholecalciferol leading to consideration of the potential rodenticidal uses (Agnew, 2010, 2011; Eason, 1991, 1993; Marshall, 1984). Cholecalciferol has several potential advantages over current rodenticides including a reduced risk to non-target species, such as birds, who possess a relatively high tolerance to the compound (Erickson and Urban, 2004; Marshall, 1984). In regards to human safety, deficiency in the compound is the prevailing problem with one billion people worldwide thought to be deficient (Holick, 2007); deficiency can lead to rickets in children and osteoporosis in adults (Feldman and Malloy, 2004; Holick, 2003, 2006).

Anticoagulant poisons, such as warfarin, are the most commonly used method of rodent control; their action inhibits the body's natural blood clotting pathways. Prolonged oral use of a rodenticide causes natural selection of warfarin resistant rats (Boyle, 1960; Lasseur et al., 2005; Lund, 1964; Pelz et al., 1995; Tanikawa et al., 2006; Thijssen, 1995) which has spawned a more potent '*second generation*' of anticoagulants including bromadiolone and difenacoum; however, resistance to these compounds has now been reported (Endepols et al., 2012; Greaves et al., 1982; Rowe et al., 1981).

A behavioural resistance has also been observed when using anticoagulant baits (Brunton et al., 1993; Macdonald et al., 1999). This behavioural resistance stems from the neophobic nature of many pest species, specifically, a hesitancy to approach unfamiliar foods and objects (Barnett, 1958; Beck et al., 1988; Sunnucks, 1998). This behaviour decreases the effectiveness of bait approaches (Brunton et al., 1993; Quy et al., 1992).

There is a need for an alternative to rodent control performed with anticoagulant bait, which differs both in active ingredient and manner of delivery. Cholecalciferol, as well as being a suitable rodenticide, can be delivered via a transdermal route (Agnew, 2010, 2011). Here we present two *in-vitro* models for the delivery of cholecalciferol through a synthetic membrane and the corresponding *in-vivo* data. The *in-vitro* models have been used to determine an optimum formulation, comprising of active

* Corresponding author. +44 (0) 121 204 3913.
E-mail address: a.j.ingham@aston.ac.uk (A. Ingham).

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ingredient, penetration enhancer and stabilising agent which promotes enhanced delivery of cholecalciferol through the dermis.

The legislative guidance on biocidal products, including rodenticides, is given in Regulation No. 528/2012 (concerning the making available on the market and use of biocidal products) (European Commission, 2012). In order for a rodenticide to be approved as a biocidal product it must first be proven '*sufficiently effective*'. The *in-vivo* investigations required by law are provided under specific guidance. To prevent replication of *in-vivo* investigations it was logical to use the guidelines suggested by the Regulations in both data generation and model development.

2. Materials and methods

2.1. Materials

European pharmacopoeia grade cholecalciferol was purchased from Fagron UK Ltd. (Newcastle on Tyne, UK). Penetration enhancers ethanol, oleic acid and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific UK Ltd. (Loughborough, UK), 2-pyrrolidone was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA); all were laboratory reagent grade. Methyl cellulose (Fisher Scientific UK Ltd. (Loughborough, UK)) and polyethylene glycol mw200 (PEG 200) (Sigma–Aldrich Co. (St. Louis, MO, USA)) were used as viscosity modifying agents. The receiver phase for the *in-vitro* studies was composed of ethanol (Fisher Scientific UK Ltd. (Loughborough, UK)), polyethylene glycol mw200 (Sigma–Aldrich Co. (St. Louis, MO, USA)) and deionised water. Regenerated cellulose dialysis membrane (visking tubing, Fisher Scientific UK Ltd. (Loughborough, UK)) was used for the synthetic membrane (Corrigan et al., 1980; Haigh and Smith, 1994; Wang et al., 2006; Wissing and Müller, 2002).

2.2. Methods

2.2.1. Permeation studies

2.2.1.1. Cellulose tubing in-vitro model. Dialysis tubing was cut into strips and sealed on one side. For each formulation 1 ml was dispensed into the dialysis tubing and placed in a 50 ml centrifuge tube containing 45 ml of receiver phase. An aqueous ethanol (10/90 (v/v)) receiver phase containing 6% (v/v) PEG 200 was used as cholecalciferol is practically insoluble in water (British Pharmacopoeia, 2013). Sampling of the receiver phase was performed every hour for the first 4 h then every 2 h for a total experiment time of 8 h. At each sampling point 5 ml of the receiver phase was removed and replaced with stock receiver phase. Of the 5 ml, 200 μl was extracted and diluted before analysis. The temperature of the receiver phase was maintained at 26 °C ± 2 °C by the immersion of the centrifuge tubes in a heated water bath. Three replicates were employed for each formulation.

2.2.1.2. Diffusion cell in-vitro model. The membrane was cut into 5 × 5 cm squares and placed between the donor and receiver chambers of a static diffusion cell (Ingham Group, Aston University, UK). For each formulation 15 ml of receiver solution was placed in to the receiver chamber while 5 ml of the assay was dispensed into the donor. An aqueous ethanol (10/90 (v/v)) receiver phase containing 6% (v/v) PEG 200 was used. Sampling of the receiver phase was performed every hour for the first 5 h then every 2 h for a total experiment time of 9 h. At each sampling point 5 ml of the receiver phase was removed and replaced with stock receiver phase. Of the 5 ml, 200 μl was extracted and diluted before analysis. The temperature of the receiver phase was maintained at 37 °C ± 2 °C with a heated stirring plate.

2.2.1.3. Formulations. Two batches of five formulations were tested with the cellulose tubing model; the first set of five formulations investigated a range of chemical penetration enhancers at various concentrations to determine the optimum chemical penetration enhancer. The second batch of five formulations investigated a range of DMSO/ethanol co-solvents.

Two batches of formulations were tested with the diffusion cell model; the first set investigated a range of penetration enhancers at various concentrations to determine the optimum chemical penetration enhancer. The second batch varied in cholecalciferol concentration.

2.2.1.4. Chemical penetration enhancement. Chemical penetration enhancers (Stoughton and Fritsch, 1964) have been shown to increase the penetration of compounds such as antiviral agents, steroids and antibiotics (Williams and Barry, 2012). DMSO and the sulfoxide family damage the stratum corneum promoting intercellular passage of the penetrant. Organic solvents, such as ethanol, increase penetration by extracting the lipids from the horny layer. Fatty acids such as oleic acid have also been shown to improve dermal penetration (Larrucea et al., 2001; Meshulam et al., 1993; Moreira et al., 2010) by creating reservoirs in the stratum corneum in which the penetrant can move through. DMSO, ethanol, water, 2-pyrrolidone and oleic acid were chosen as the potential penetration enhancers.

2.2.1.5. Membrane preparation. Cellulose membranes were cleaned before use in 1 l of cleaning solution consisting of 2% (w/v) sodium bicarbonate (Sigma–Aldrich Co. (St. Louis, MO, USA)) and 1 mM of ethylenediaminetetraacetic acid (EDTA) in distilled water. The cellulose membrane was placed in the solution while the temperature was brought up to 80 °C; the solution was then maintained at 80 °C for 30 min. After cleaning, the membranes were rinsed thoroughly with distilled water and kept in a bath of distilled water for a maximum of 5 days prior to use in accordance with the manufacturer's guidelines (Medicell International Ltd., 2004).

2.2.1.6. Data analysis. For each formulation used in the *in-vitro* study, the drug flux (J_s) was calculated. This value was obtained with Fick's first law of diffusion after plotting the cumulative drug amount in the receiver chamber against time (Martin and Bustamante, 1993):

$$J_s = \frac{1}{A}\left(\frac{dM}{dt}\right)_{ss}$$

where A refers to the cross sectional area (cm^2), and $(dM/dt)_{ss}$ is the rate of drug permutation across the membrane over time (mg/h) at steady state. By plotting the cumulative amount of drug received in the receiver chamber against time $(dM/dt)_{ss}$ (the gradient) can be calculated at steady state (i.e. when the relationship is linear). A diffusion area of 40 cm^2 was used for the cellulose tubing calculations while an area of 2.54 cm^2 was used for the diffusion cell method.

2.2.1.7. HPLC analysis. Quantitative analysis of all cholecalciferol assays was performed with high performance liquid chromatography (Prominence Modular HPLC (Shimadzu Corporation, Japan)) using a UV diode array. The specific wavelength of detection was 265 nm. A Luna 3μ NH_2 100A column (Phenomenex, Cheshire, UK), dimensions: 150 × 4.6 mm was used with a NH_2 3 mm ID security guard and holder. The total flow rate was 2 ml/min and the run time was 6 min. The mobile phase consisted of hexane/isopropanol (HPLC grade). During the first 3 min of the run a gradient ratio of 99/1–50/50 (v/v) hexane/isopropanol was used, a ratio of 99/1 (v/v) hexane/isopropanol was

used for the remainder of the run. The concentration of cholecalciferol was calculated for each assay by using an equation derived from the slope of the standard curve prepared for cholecalciferol ($r^2 = 0.999$) at 265 nm.

2.2.2. In-vivo *studies*

2.2.2.1. Animal husbandry. All *in-vivo* investigations were undertaken at Cellvax Pharma (Paris, France). The experimental protocols were approved by the Ministere de L'enseignement Superieur de la Recherche (ComEth Anses/ENVA/UPEC 16). Male Sprague-Dawley rats (Harlan, France) aged between 7–10 weeks were employed in the protocol. Each rat weighed between 250 and 350 g and was of SOPF (Specific and Opportunistic Pathogen Free) status. Animals were housed in polyethylene cages in a climate and light controlled environment. Hours of lighting were between 7:00 and 19:00, the temperature and humidity was maintained at 21 ± 1 °C and 70% RH, respectively. Animals had a constant supply of food and water. In the case of the initial chemical penetration enhancement study the animals were housed singularly, in the efficacy screening study animals were housed in groups of twos and threes, fur shaving was employed to identify each animal only. All animals were acclimatised to the laboratory for at least one week prior to the start of the experiment.

2.2.2.2. Distress scoring. In order to assess the effect of the formulation on the animals, and to quantify any distress or suffering, an assessment of distress was made 2–3 times a day using a distress scoring chart. The chart considered five key areas in which distress could be displayed, these areas are: general appearance, appearance of application site, natural behaviour, provoked behaviour and food and water intake. Each of these areas was given a mark out of 3 providing for a maximum distress of 15. A score of 0 would suggest the formulation had no effect. A score of 1–5 is indicative of minor changes in behaviour or appearance; a score of 5–10 is suggestive of moderate changes while a score of over 10 suggests significant changes in behaviour and appearance including pilo-erection or comatose state.

2.2.2.3. Protocol 1 (screening test and efficacy evaluation). The screening test protocol is based upon the guidelines set out by the European Plant Protection Organisation (EPPO), specifically guidelines PP 1/113(2) (EPPO, 1998). For the testing of a novel rodenticide; screening tests are suggested in which five male laboratory strains of either *Rattus norvegicus* or *Mus musculus* are used.

The procedure for the application of the transdermal rodenticide was based upon the Organisation for Economic Co-operation and Development (OECD) guidelines 434 (OECD, 2004) in which the formulation is applied to a 10 cm^2 area on the scruff of the animal. Mortality and time until endpoint was recorded for each formulation. Each formulation was dosed to 5 animals. A volume of 1 ml was dosed to each animal. While this protocol advises to shave the area of application site these measures were not incorporated in the current study as an '*in-use*' application was preferred.

2.2.2.4. Data analysis. In order for a rodenticide to be considered for rodent control it must be proven to be '*sufficiently effective*'. 100% mortality during screening is considered to be effective (EPPO, 1998).

Animal distress was monitored 3 times daily during a 5 day period, animals were then monitored 2 times per day for a further 9 days. Formulations which exhibit less than 100% mortality were regarded as ineffective. The distress of the animal during the protocol was monitored using a distress scoring chart proposed by Wolfensohn and Lloyd (2003), with amendments to enhance description under provoked response.

Weight data was collected for all animals.

2.2.3. Statistical analysis

Statistical analysis of the *in-vitro* data was performed using a one-way analysis of variance (ANOVA); this was to determine statistical differences between the drug fluxes determined for each formulation. For the *in-vivo* data set one-way ANOVAs were performed on the time till mortality and the average distress at endpoint. In cases where ANOVA suggested significant differences, Tukey's multiple comparison tests were performed between formulations to determine the specific pairing in which differences were observed. All one-way ANOVAs and Tukey multiple comparisons were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla California USA, www.Graphpad.com). A probability, $p < 0.1$, was considered significant.

3. Results

3.1. Permeation results

3.1.1. Cellulose tubing in-vitro *model*

3.1.1.1. Chemical penetration enhancement. Cholecalciferol permeation profiles using: DMSO, ethanol, oleic acid, 2-pyrrolidone and water as penetration enhancers were obtained through cellulose tubing in an *in-vitro* model and are illustrated in Fig. 1. Fig. 2 shows the drug flux comparisons between the formulations tested. The 90/10 (v/v) DMSO/ethanol (0.26 ± 0.044 mg/cm^2 h) formulation was shown to significantly increase ($p < 0.1$) the drug flux of cholecalciferol when compared to the ethanol (0.14 ± 0.008 mg/cm^2 h) and 2-pyrrolidone (0.13 ± 0.038 mg/cm^2 h) formulations. Based on these results, the DMSO/ethanol co-solvent was selected for further investigations as a penetration enhancer for cholecalciferol.

3.1.1.2. DMSO/ethanol co-solvent penetration enhancement. The permeation profiles of cholecalciferol using DMSO/ethanol co-solvents are shown in Fig. 3. The results suggest no statistically significant differences (one-way ANOVA, $p < 0.1$) between the drug flux of cholecalciferol with a DMSO/ethanol co-solvent across the range 50/50 (v/v)–90/10 (v/v), respectively.

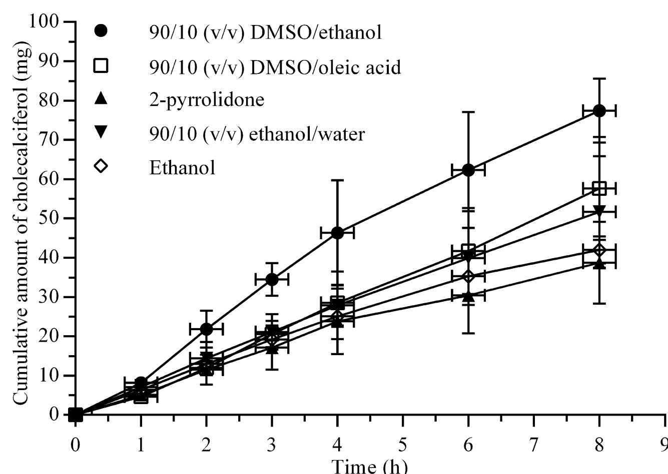

Fig. 1. Cholecalciferol permeation profiles obtained with the cellulose tubing *in-vitro* model. All formulations contained 10% (w/v) cholecalciferol released from a volume of 1 ml. Mean values ± SD ($n = 3$).

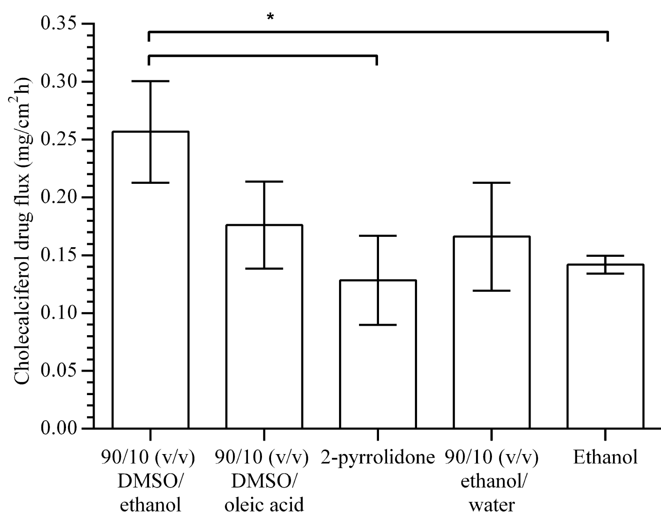

Fig. 2. Drug flux comparisons for chemical penetration enhancement of cholecalciferol obtained with the cellulose tubing *in-vitro* model. Mean values ± SD. Significant differences where indicated ($p < 0.1$, Tukey's multiple comparison test) ($n = 3$).

3.1.2. *Diffusion cell* in-vitro *model*

3.1.2.1. Chemical penetration enhancement. Fig. 4 shows the permeation of cholecalciferol through a synthetic membrane using the diffusion cell arrangement. Based on the results from the cellulose tubing *in-vitro* model, formulations containing high percentages of DMSO were investigated, while ethanol formulations served as comparators. A combination of cholecalciferol concentrations and chemical penetration enhancers were used.

The results suggest that for both cholecalciferol concentrations (20% and 40% (w/v)) the ethanol (2.23 ± 0.048 mg/cm^2 h and 6.62 ± 0.44 mg/cm^2 h) formulations significantly increased ($p < 0.1$) drug flux when compared with a DMSO/ethanol co-solvent (1.19 ± 0.078 mg/cm^2 h and 4.78 ± 0.062 mg/cm^2 h). A summary of cholecalciferol drug fluxes obtained with each penetration enhancer is contained in Table 1.

3.1.2.2. Dose response. Fig. 5 suggests the dose response relationship as determined by the diffusion cell *in-vitro* model A range of cholecalciferol concentrations were investigated

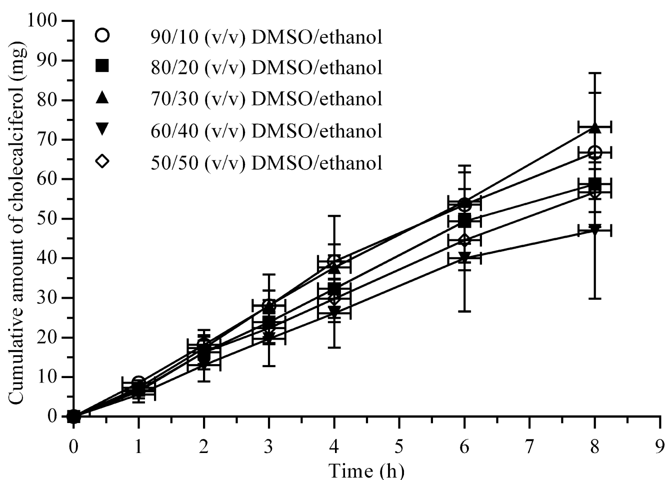

Fig. 3. Cholecalciferol permeation profiles using DMSO/ethanol co-solvents obtained with the cellulose tubing *in-vitro* model. All solutions contain 10% (w/v) cholecalciferol released from a 1 ml volume. Mean values ± SD ($n = 3$).

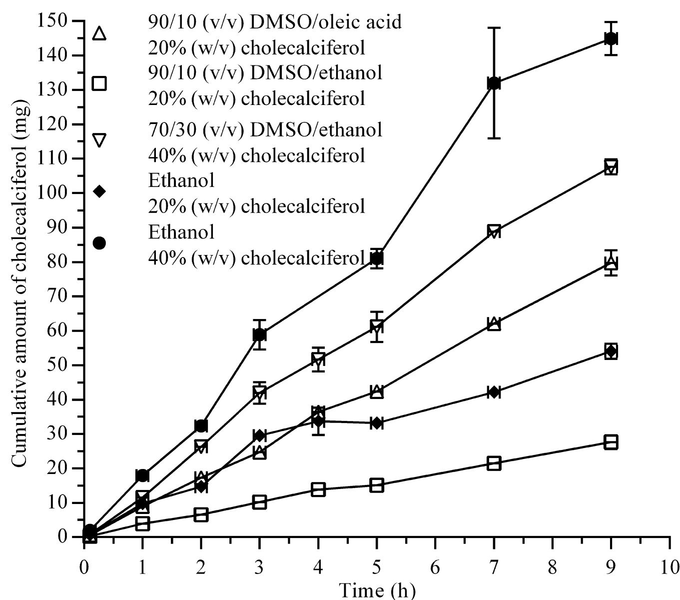

Fig. 4. Cholecalciferol permeation profiles obtained with the diffusion cell *in-vitro* model. A volume of 5 ml was used for the donor phase. Dashed lines represent the linear regression for the line of best fit. Mean values ± SD ($n = 3$).

comprising of 20%, 9%, 1.5% and 0.15% (w/v). The concentrations were taken from the fixed dose procedure for determining oral toxicity (OECD guidelines 420) (OECD, 2001). The results suggest that the 20% (w/v) cholecalciferol concentration (4.97 ± 0.18 mg/cm^2 h) produced a significantly greater ($p < 0.1$) drug flux when compared to the other tested concentrations. A non linear relationship was observed between formulation drug flux (mg/cm^2 h) plotted against cholecalciferol concentration (w/v%) ($y = 0.997 \ln(x) + 1.49$, $r^2 = 0.91$, Microsoft Excel version 2003).

3.2. In-vivo *results*

Two *in-vivo* investigations were conducted: a screening protocol, used to compare the range of penetration enhancers and validate the *in-vitro* models, and an additional screening protocol, used to determine if the favoured formulation was '*sufficiently effective*' as defined by the Regulation.

3.2.1. Protocol 1 (screening test)

A total of five formulations were implemented in the screening protocol comparing penetration enhancers. These formulations demonstrated high drug flux when compared to other chemical penetration enhancers during *in-vitro* investigations. Two of the formulations used a DMSO/ethanol co-solvent, as indicated by the cellulose tubing model; two of the formulations used ethanol as indicated by the diffusion cell model while the remaining formulation consisted of DMSO and oleic acid, as highlighted in both *in-vitro* models. Two variations of cholecalciferol concentration were implemented; 40% (w/v) in ethanol and 70/30 (v/v) DMSO/ethanol co-solvent, while 20% (w/v) was used with ethanol, 90/10 (v/v) DMSO/ethanol and 90/10 (v/v) DMSO/oleic acid. A thickening agent (methyl cellulose) was added to all formulations containing DMSO, 1% (w/v) was added to the 90/10 (v/v) DMSO/ethanol and the 70/30 (v/v) DMSO/ethanol formulations, while 0.75% (w/v) was added to the 90/10 (v/v) DMSO/oleic acid formulation.

Fig. 6 shows the respective survival rates for each of the five formulations designed to improve the transport of cholecalciferol through rat skin. The results suggest that only formulations which

Table 1
Formulation parameters and drug flux. Drug flux quoted as mean values ± SD ($n = 3$).

	Cholecalciferol concentration % (w/v)	Solvent	Diffusion area (cm^2)	Drug flux (J_s) (mg/cm^2 h)
In-vitro model: *cellulose tubing*				
Comparison of chemical penetration enhancers	10	90/10 (v/v) DMSO/ethanol	40	0.26 ± 0.044
	10	90/10 (v/v) DMSO/oleic acid	40	0.18 ± 0.038
	10	90/10 (v/v) ethanol/water	40	0.17 ± 0.047
	10	Ethanol	40	0.14 ± 0.008
	10	2-pyrrolidone	40	0.13 ± 0.038
DMSO/ethanol co-solvent penetration enhancement	10	70/30 (v/v) DMSO/ethanol	40	0.23 ± 0.027
	10	90/10 (v/v) DMSO/ethanol	40	0.22 ± 0.049
	10	80/20 (v/v) DMSO/ethanol	40	0.19 ± 0.03
	10	50/50 (v/v) DMSO/ethanol	40	0.18 ± 0.025
	10	60/40 (v/v) DMSO/ethanol	40	0.16 ± 0.054
In-vitro model: *diffusion cell*				
Comparison of chemical penetration enhancers	40	Ethanol	2.54	6.62 ± 0.44
	40	90/10 (v/v) DMSO/ethanol	2.54	4.78 ± 0.062
	20	90/10 (v/v) DMSO/oleic acid	2.54	3.5 ± 0.15
	20	Ethanol	2.54	2.23 ± 0.048
	20	90/10 (v/v) DMSO/ethanol	2.54	1.19 ± 0.078
Dose–response	20	50/50 (v/v) DMSO/ethanol 15% (v/v) PEG 200	2.54	4.97 ± 0.18
	9	50/50 (v/v) DMSO/ethanol 15% (v/v) PEG 200	2.54	3.14 ± 0.18
	1.5	50/50 (v/v) DMSO/ethanol 15% (v/v) PEG 200	2.54	0.84 ± 0.037
	0.15	50/50 (v/v) DMSO/ethanol 15% (v/v) PEG 200	2.54	0.09 ± 0.018

contained DMSO produced 100% mortality. In line with the EPPO guidelines these formulations can be considered for further investigation. The ethanol formulations containing 20% and 40% (w/v) cholecalciferol caused 20% and 60% mortality, respectively; these formulations would be ineffective as a rodenticide and warranted no further investigation. The results suggest that the inclusion of DMSO has improved the penetration of cholecalciferol causing 100% mortality within 5 days of application when compared with the ethanol formulations. No significant differences ($p < 0.1$) were found between the times taken for mortality across the three formulations that exhibited 100% mortality.

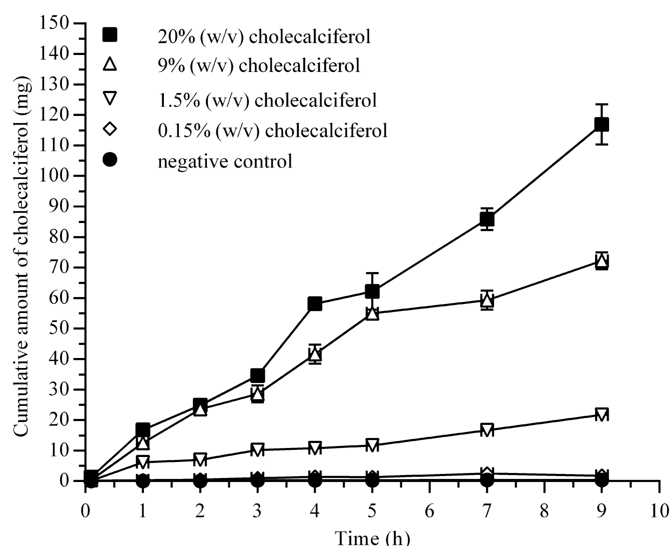

Fig. 5. Cholecalciferol permeation profiles using varied concentrations of cholecalciferol obtained with the diffusion cell *in-vitro* model. A total of 5 ml was added to each donor chamber. All formulations had the indicated amount of cholecalciferol in a formulation consisting of 15% (v/v PEG 200) made up to volume using a 50/50 co-solvent of DMSO/ethanol. Mean values ± SD ($n = 3$).

Fig. 7 shows the quantification of distress as obtained with the distress scoring chart. The results suggest the average distress at the endpoint ranged between 5 and 10, apart from the 20% (w/v) cholecalciferol in ethanol which had an average distress of 2; however this formulation only produced 20% mortality. No significant differences ($p < 0.1$) in distress at endpoint were observed between formulations which caused 100% mortality. The results suggest that only moderate changes in behaviour are exhibited by a rodent prior to mortality including a reduction in mobility and a reduced food and water intake.

3.2.2. Protocol 1 (efficacy evaluation)

An additional formulation was used for the determination of efficacy consisting of 50/50 DMSO/ethanol, 15% (w/v) PEG 200 and 20% (w/v) cholecalciferol. A negative control was also employed consisting of 15% (v/v) PEG 200 in a 50/50 DMSO/ethanol co-solvent. The co-solvent penetration enhancer of DMSO/ethanol was used as the result from the screening protocol suggested that this formulation warranted further investigation. The addition of PEG 200 to the formulation was designed to thicken the formulation and aid in adherence to the unprepared coat of a rat. A 50/50 (v/v) DMSO co-solvent also provided improved physical properties when compared to the 90/10 (v/v) DMSO/ethanol co-solvent specifically a reduced freezing point.

Fig. 8shows the survival analysis of the efficacy screening evaluation. A 100% mortality was achieved with a 50/50 DMSO/ethanol, 15% (w/v) PEG 200 and 20% (w/v) cholecalciferol formulation suggesting that the formulation would be sufficiently effective for consideration as a rodenticide. The formulation also exhibited a

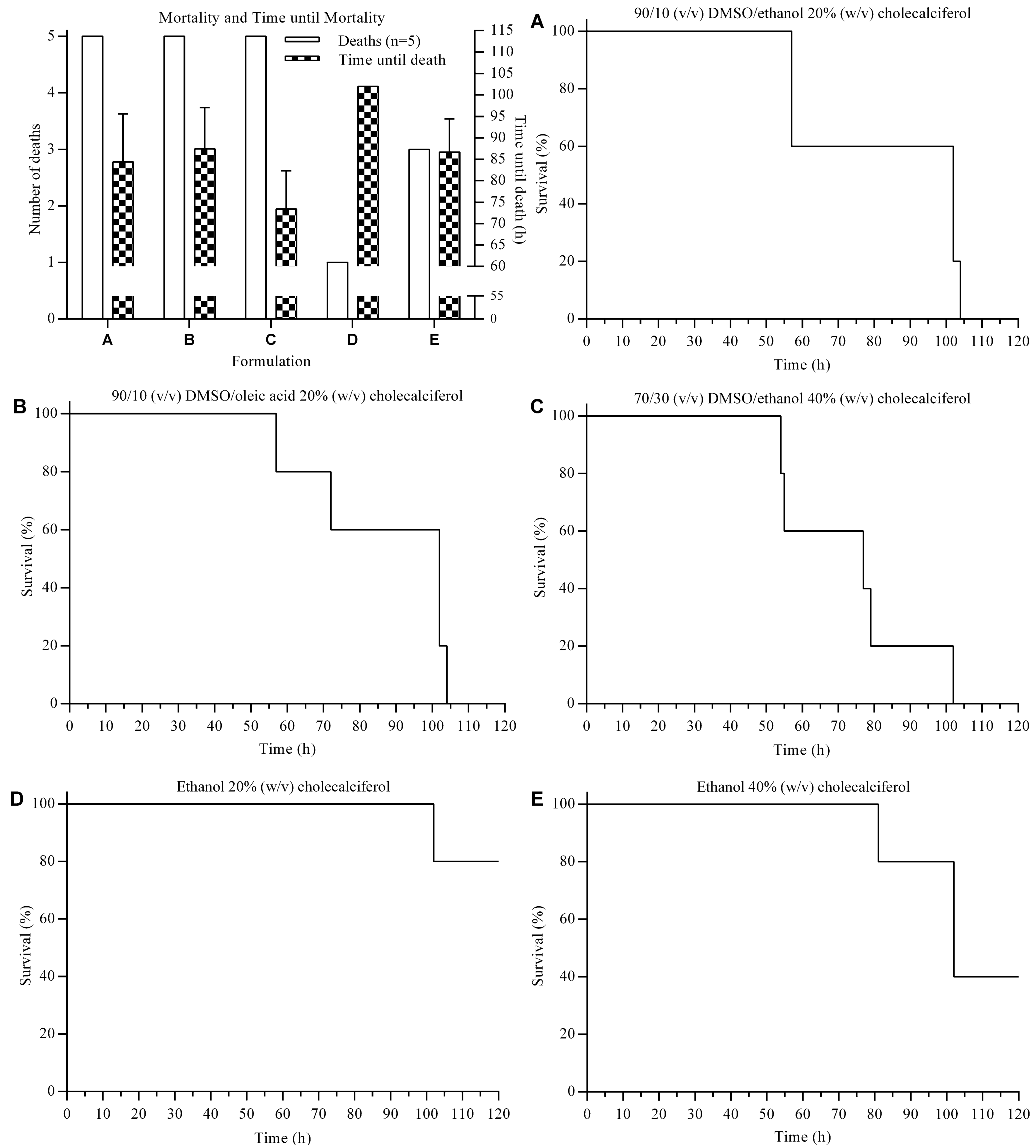

Fig. 6. Mortality and time until endpoint summary for formulations. (A–E) Survival rates for transdermal cholecalciferol formulations. Mean values ± SD ($n = 5$).

reduced time to mortality when compared to the DMSO/ethanol co-solvents dosed in the screening protocol (90/10 (v/v) DMSO/ethanol 20% (w/v) cholecalciferol = 84 h ± 25 h, 50/50 (v/v) DMSO/ethanol 20% (w/v) cholecalciferol = 64 h ± 22 h). The negative control did not yield any fatalities thus it can be concluded that the cholecalciferol is responsible for the lethal action.

Fig. 9 shows the distress scoring chart and animal weight for the efficacy evaluation and negative control. The negative control showed no changes in behaviour, coupled with an increase in weight suggesting that food and water intake was healthy. The 20% (w/v) cholecalciferol concentration exhibited significant changes in behaviour, however only minor changes were observed during the first day followed by a quick increase in distress as recorded within the distress scoring chart.

4. Discussion

When evaluating rodenticidal formulations, the European Union requests *in-vivo* data in the form of screening tests, efficacy evaluations and field trials; however, *in-vitro* data plays a vital role in determining appropriate formulations to take forward into these tests.

In this research two *in-vitro* models were used to compare formulations developed internally with those previously speculated. A drug flux of 0.26 ± 0.044 mg/cm^2 h was determined for cholecalciferol dosed in a DMSO/ethanol co-solvent (10% (w/v) cholecalciferol) by using a cellulose tubing model. The ethanol formulation proposed by Agnew (2010, 2011) produced a lower drug flux of 0.13 ± 0.0038 mg/cm^2 h (10% (w/v) cholecalciferol) (Fig. 2). The opposite was observed when these formulations were tested using the diffusion cell model (Fig. 4), the model suggested higher drug flux for ethanol formulations when compared to DMSO/ethanol co-solvent over two cholecalciferol concentrations (Table 1). All penetration enhancers tested with both models showed inflated drug fluxes when evaluated with the diffusion cell model.

The most notable differences between the *in-vitro* models is increased concentration of the solute in the diffusion cell formulations and the absence of donor phase stirring in the cellulose tubing model, potentially leading to a static solvent layer

Fig. 7. Average distress at mortality. (A–E) Formulation humaneness as recorded with the distress scoring chart. Mean values ± SD (n = 5).

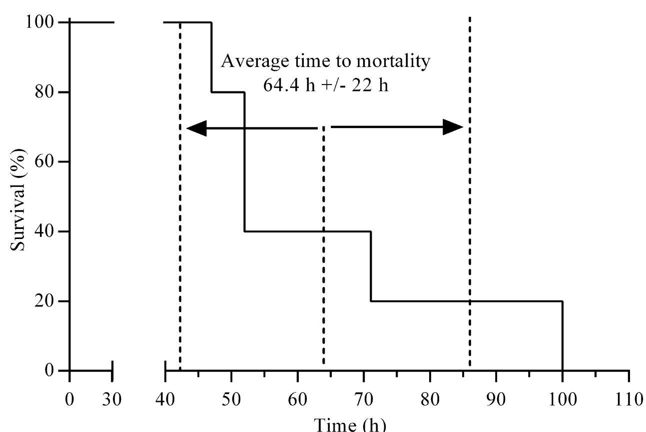

Fig. 8. Survival analysis for the efficacy evaluation screening test on formulation: 20% (w/v) cholecalciferol and 15% (v/v) PEG 200 in 50/50 (v/v) DMSO/ethanol (n = 5).

between the donor phase and the synthetic membrane. The model thus takes the form of a multilayer arrangement which can be described mathematically as (Martin and Bustamante, 1993):

$$J_s = \frac{1}{A}\left(\frac{dM}{dt}\right) = \frac{D_m K D_a C_1}{h_m D_a + 2 h_a D_m K}$$

In which D_m and D_a are the membrane and static layer diffusivities, respectively, and K represents the partition coefficient. It can also be seen that the concentration of the solute in the donor phase C_1, membrane thickness h_m and the static diffusion layer thickness h_a all contribute to the drug flux calculation. Fundamentally, an increase in solute concentration in the donor phase will increase flux, while an increase in the thicknesses of the membrane and static diffusion layer will decrease flux. The static diffusion layer thickness h_a is a physical property and is proportional to the amount of agitation (Martin and Bustamante, 1993), greater stirring reduces h_a promoting higher fluxes which can be seen when comparing the two *in-vivo* models in this research.

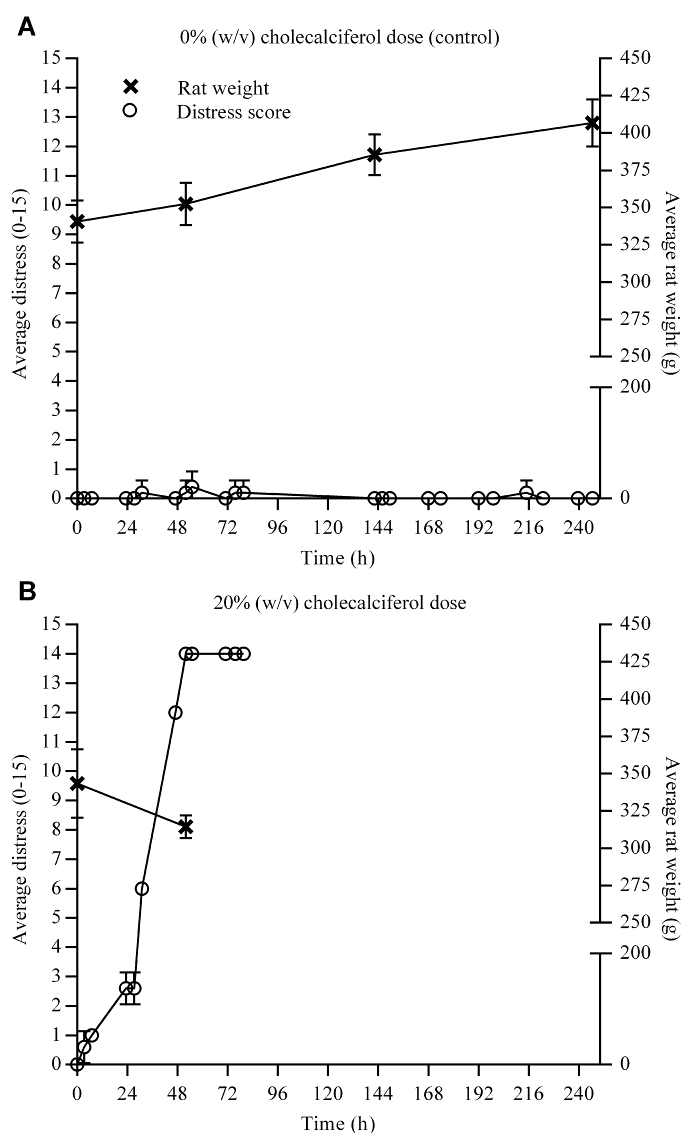

Fig. 9. Formulation humaneness as recorded with the distress scoring chart and weight. Mean values ± SD ($n = 5$). Formulations contained indicated amounts of cholecalciferol in 50/50 (v/v) DMSO/ethanol co-solvent containing 15% (v/v) PEG 200.

This would suggest DMSO ethanol combinations are better able to disrupt static diffusion layers than the ethanol solvent system. Molecular interaction of the co-solvent could be thought to be further enhancing dissolved solute movement in the donor phase. This action could be likened to the increase in solubility of components through molecular interactions of co-solvents.

The screening protocol served to both compare DMSO co-solvents with ethanol formulations and determine whether these formulations warranted further investigations. Ethanol formulations containing 20% and 40% (w/v) cholecalciferol exhibited 20% and 60% mortality, respectively, during the screening protocol; while all formulations containing DMSO displayed 100% mortality (Fig. 6). In line with the EPPO guidelines (EPPO, 1998) the ethanol based formulations would not be considered for use as a rodenticide as they do not produce sufficient mortality. The difference between these investigations and that of Agnew (2010, 2011) may lie in the origin of the rodent. In this study, Sprague-Dawley rodents were used with weights between 250 and 350 g whereas a wild Norway rat of unknown weight was used in Agnew's work. Potential differences in weight may have resulted in the more potent dose during Agnew's own study.

A second *in-vivo* screening test was employed to further investigate a DMSO/ethanol co-solvent with a reduced level of DMSO as the *in-vitro* investigations suggested no significant difference in drug flux when the ratio of DMSO/ethanol was varied (Fig. 3). A negative control, containing no cholecalciferol, was also employed during this study to confirm that mortality could be attributed to the cholecalciferol. The 20% (w/v) cholecalciferol concentration in 50/50 DMSO/ethanol and 15% (v/v) PEG 200 resulted in 100% mortality suggesting that this concentration would be sufficiently effective for further regulatory trials. The 1 ml dose produced 100% mortality in 64.4 h ± 22 h; quicker than that found in the previous screening study (Figs. 6 and 8). No adverse effects were seen in the animals dosed with the negative control.

The *in-vivo* responses correlated better with the cellulose tubing *in-vitro* model suggesting formulations applied direct to the fur of rats skin display absorption controlled by a static diffusion layer. DMSO and oleic acid have not been previously tested with cholecalciferol, they have however, been shown to improve penetration (Williams and Barry, 2012) of compounds such as anti-inflammatory drugs (Gwak and Chun, 2002; Larrucea et al., 2001; Meshulam et al., 1993; Moreira et al., 2010). While DMSO is proven to disrupt the dermis it is not typically used as it causes an unpleasant taste in the mouth of humans and has long lasting effects on the area of skin to which it is applied. In the context of its use as a rodenticide these would be disregarded. In this regard, both permeation studies and *in-vivo* results suggest that a DMSO/ethanol co-solvent is the most effective penetration enhancer investigated for use in combination with cholecalciferol.

The results suggest that transdermal delivery of cholecalciferol has the potential to provide an alternative to anticoagulant baiting methods. While baiting approaches have been used successfully they depend on the rodent ingesting lethal amounts of the bait. This ingestion is not guaranteed and is a contributor to the development of anticoagulant resistance (Quy et al., 1992). A one dose transdermal application is the most efficient manner in which to deliver the toxin; with an optimised 'minimum' dose been administered reducing the waste and any unnecessary environmental exposure. The difficulty associated with direct application of a rodenticide have been discussed and details for successful deployment provided elsewhere (Goode, 2010). Transdermal delivery of cholecalciferol is a feasible alternative to anticoagulant baits, however larger efficacy evaluations are required to produce statistically significant data for the European Union as well as proof of this formulations effectiveness in the field.

5. Conclusions

A suitable chemical penetration enhancer to facilitate transdermal delivery of relatively low levels of cholecalciferol has been found.

Through both *in-vitro* and *in-vivo* investigations a 1 ml dose of 50/50 (v/v) DMSO/ethanol, 15% (v/v) PEG 200 vehicle containing 20% (w/v) cholecalciferol has been proven to be sufficiently effective as a rodenticide (Regulation No. 528/2012). This dose was found to cause 100% mortality in 64.4 h ± 22 h.

Two *in-vitro* models have been developed to screen potential formulations. The cellulose tubing model has correlated with *in-vivo* investigations of rat skin suggesting that future work should consider the use of static diffusion layers in modelling formulations of this type.

Transdermal delivery of cholecalciferol has been shown to be sufficiently effective in screening studies, thus offering an alternative to anticoagulant baits that differs in both active ingredient and the manner of its controlled delivery.

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