Characterisation of Actions of p-menthane-3,8 diol (PMD) Repellent Formulations against *Aedes aegypti* mosquitoes

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Abstract

Background

Characterisation of mosquito repellents using arm-in-cage tests are performed by assessing the ED_{95}, half-life and complete protection times (CPT). This study fully characterises p-menthane-3,8 diol PMD which has not been widely studied, and a long-acting formulation containing a PMD-vanillin composite.

Method

A series of arm in cage tests against *Aedes aegypti aegypti* (Dipera-Culicidae) strain mosquitoes were devised using 6 volunteers to estimate CPT or 10 to estimate the effective dose (ED_{95}) and half-life for three repellents: 20% DEET, 30% PMD and a novel 30% PMD-vanillin formulation. Non-linear Regression analysis was used to characterise the relationship between applied dose and CPT. were also estimated

Results

PMD and DEET showed a very similar log dose relationship to CPT; however, the PMD-vanillin formulation exhibited a sigmoidal 'S-shape' relationship. This resulted in a 1.5 times higher CPT for PMD-vanillin compared to that of 20% DEET when applied at a dose of 1.6mg/cm^2, but little difference at lower doses of 0.8-1mg/cm^2. The ED_{95} value for the 30% PMD and PMD-vanillin formulations were 0.25 and 0.24 mg/cm^2 respectively, these being higher than that for 20% DEET (0.09 mg/cm^2). The half-lives for 30% PMD and 20% DEET were similar (2.23 *vs.* 2.74 hrs.), but longer for the PMD-vanillin formulations (3.8 hrs.).

Conclusion

Such a full characterisation for other repellent formulations, particularly those claiming extended longevity, should be conducted in order to identify differences at various applied doses.

Keywords

MOSQUITO, REPELLENT, DEET , PMD, HALF LIFE, LONGEVITY,

Introduction

Assessing and comparing the efficacies of mosquito repellent formulations is an important feature of repellent research which has direct applications in the selection of products by the user. The mathematical models for characterising repellents were first developed in the 1970s and 1980s through the pivotal work of Rutledge[1] and Bueschner et al.[2]. They identified three principal parameters using cage tests, with which a formulation could be assessed: the effective dose required to repel up to 95% of mosquitoes (ED_{95}), the rate of decline in repellent after a dose greater than the ED_{95} is applied (the half-life), and the time that protection of > 95% is retained after applying a given dose of repellent. They used N,N-diethyl-3-methyl benzamide (DEET) as the standard repellent, and compared it to others available at the time, none of which are actually now used in commercial products. The cage tests they used were also quite different in design to those currently described by the World Health Organisation (WHO)[3], which is endorsed as the standard by most manufacturers and regulatory authorities. Essentially, the earlier cage tests used only 10-20 mosquitoes [1,2] introduced to a small demarcated area of human skin pre-treated with repellent. Modern cage tests use quite large cages with up to 200 mosquitoes exposed to the whole forearm of a volunteer.[3] Field tests are also widely employed in repellent efficacy evaluation but are not considered in this paper.

The ED_{95} index provides an indication of the potency of a repellent and should be lower than the dose readily achievable by the user. Most commercial products contain one of four active ingredients: DEET, 2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester (picaridin), *p*-menthane-3,8-diol (PMD) or ethyl butylacetyl-aminopropionate (IR3535), all of which are claimed to have an effective ED_{95} value against most mosquito species, though in some cases there is not a great deal of evidence for this in the public domain. Accepting that the user will apply a dose to provide 100% protection, the next critical question is how long that level of protection will be retained, a concept known as the complete protection time (CPT). The early work by Beuscher[2] indicated a logarithmic relationship between increasing dose and protection time. This relationship is important, since above a particular dose of repellent (depending on the active ingredient concentration), further incremental doses offer less advantage in terms of protection time. The CPT is usually assessed as the Time to First Bite (TFB) when an arm is introduced into a cage of mosquitoes. An important realization is that such CPTs should not be taken as a reflection of the actual protection time that a user might experience in the field and are only useful in comparing durations of protection of different repellents when assessed under exactly the same laboratory conditions. For this reason, DEET is used as a 'gold standard' for such cage tests.

We have found little in the public domain that has fully characterised formulations of PMD to asses ED_{95}, half-life and CPT values at different doses of repellent. Carroll described 3 laboratory and 4 previous field studies at fixed doses to determine CPT for PMD [4] and we have identified only one measuring the other parameters.[5] The aim of this study is to fully characterise the activity of DEET against these three parameters, and compare it to PMD. Furthermore, a new PMD formulation that gives rise to a product that has been shown to provide a longer CPT than equivalent doses of DEET against *Aedes* mosquitoes will also be similarly characterised.

Experimental study design

Studies were conducted in two stages; in the first, 20% DEET and 30% PMD (both in ethanol) were compared, and in the second, 20% DEET and a long acting formulation of 30% PMD which also contained vanillin (PMD-XL, Neo-Innova) were comparatively evaluated. The design of the study was adapted from the methodologies described by the WHO.[3] The major adaptation to these guidelines is that it does not describe measurements for obtaining persistence (half life) in cage tests only for field trials. Further it describes the measurement of CPT at a single fixed dose rather than assessing a range of doses as in this study. Other adaptations were made to cage sizes and mosquito numbers in order to facilitate observations.

Each study took place over 3 months at Ross Lifescience laboratory using laboratory bred, 5-7 days old., Aedes aegypti aegypti (Dipera-Culicidae) strain mosquitoes . Laboratory environmental conditions were 27(±2)°C and 65(±5)% relative humidity. Participants occupied the laboratory only for the duration their own test exposure. Participants abstained from using scented products, smoking or drinking alcohol on testing days. The repellents investigated were DEET 20% (Alkyl Amines Chemicals Ltd*)*, PMD (Citriodiol® - *eucalyptus citriodora* oil, hydrated, cyclized) in ethanol (30% w/v) and a repellent containing 30%PMD with 5% vanillin (PMD-XL).

In preparation for the laboratory studies, the test area of volunteers' skin was washed with unscented soap and rinsed with water, rinsed with a solution of 70% w/v ethanol or isopropyl alcohol in water, an dried with a towel. Participants covered any exposed skin with gloves and long sleeves. Blinding of observers to the different classes of repellent tested could not be used in view of the very distinctive odours of the products.

A repellent or control solution (ethanol) was applied to 600 cm^2 skin surface areas on the forearms between the crease of the elbow and wrist of each of the participants. The test substance was weighed on a tared container and applied directly from the sample container onto the skin. This was evenly applied to the forearm of the participant by a member of the study staff in order to ensure complete coverage using two fingers of a gloved hand.

Time to first Bite (TFB) Studies

These were performed using a square cage (40 cm^2 each side) containing 50 mosquitoes, with six volunteers (3 male and 3 female). Landing/probing and bites on the arms of the volunteers were counted by a continually observing research assistant.

Each exposure period began with a verification of mosquito avidity by exposing the test area of the untreated control arm for 30 seconds, and the test proceeded if greater than 10 landings were observed. A measured amount of 0.2 grms of the repellent was applied to the other forearm, and this was introduced into the cage for 30s before withdrawing. This was repeated every 30 minutes until the First Confirmed Bite (FCB) was observed. The arm was then washed and swabbed with ethanol before a further increment of 0.2 g was introduced after 30 min for the same time period. The procedure was repeated until the the time to FCB was obtained for each incremental dose to a maximum of 1grm. At each increment, avidity for feeding was assessed using the control arm.. For PMD-XL, incremental doses of 0.1 g were applied. The FCB refers to a bite followed by an additional bite during one exposure period, or when one bite occurs in such an exposure period and another in the next sequential exposure period, the time period to achieve a FCB is

referred to as the complete protection time (CPT). Once a FCB occurred on a human subject, they did not participate in any additional exposure periods on that testing date.

Effective dose and half-life studies

These were performed using a cage with sides of of one cubic meter volume containing 100 mosquitoes, using 10 volunteers (5 male and 5 female).

Assessing the effective dose (ED)

A control forearm was introduced into the cage and the number of mosquitoes that landed on and/or commenced to probe the skin during a 30-second period was counted and the arm withdrawn. The other arm was treated with 0.1 g of repellent over a 600 cm^2 area, and then introduced into the cage where the landings were again counted. If the number of landings was too high to count accurately, a series of three readings of five seconds duration was taken to estimate the mean landing numbers that occurred within a 30 second period. The arm was then withdrawn and the next incremental dose tested, alternating each time with introduction of the control arm to obtain numbers landing in the absence of repellent. Incremental doses were obtained by applying a further 0.05 g dose to the treated forearm. This procedure was repeated until 100% repellence, i.e. no landings, was achieved.

Decay in repellence

The final 100% repellence dose was left on the test arm and then reintroduced into the cage after 30 minutes, landing/probing counts being obtained for 30 secs before being withdrawn. The un-treated arm was introduced into the cage and the number of landings/probings in 30 seconds was counted. This procedure was repeated every 30 minutes for up to 7 hours, using the method described earlier if landing rates were too high to estimate accurately. Protection at each 30 minute interval was calculated by the formula:

Protection = (landings on control arm-landings on treated arm)/ landings on control arm

Statistical Analyses

The CPT dose response curve was estimated by plotting dose of product against TFB and decay by plotting protection against time using non-linear regression modelling on XLSTAT 2016. Effective dose (ED95) was calculated by Probit regression analysis on SPSSv25. Comparison of CPT at fixed doses of repellents was compared using Kaplan Mieir analysis on SPSSv25

Results

Complete Protection Times

The following non-linear regression equations where obtained for 30% PMD and 20% DEET for complete protection time (CPT, minutes) plotted against concentration of repellent applied (mg/cm^2):

$CPT = 341.64*\log_{10}(mg/cm^2)+279.24$ …. 30% PMD ($R^2 = 0.88$)

$CPT = 347.24*\log_{10}(mg/cm^2)+252.28$ … 20% DEET ($R^2= 0.91$)

Figure 1 illustrates the plot of CPT versus dose for 30% PMD and 20% DEET based upon the regression equations.

For the 30% PMD-XL and 20% DEET treatments, the following regression equations were returned:

$CPT = 678.42/(1+EXP(5.03-5.64*$ dose $m/cm^2))$… 30% PMD-XL ($R^2 = 0.98$)

$CPT = 481.96*Log10$ (dose mg/cm^2)+ 340.47 … 20% DEET ($R^2= 0.94$)

Figure 2 illustrates the plot of CPT versus dose for 30% PMD-XL and 20% DEET based upon these regression equations.

At $0.8mg/cm^2$ mean CPT for 20% DEET = 300 mins (Std = 0) and for PMD-XL = 250 (Std 10) , p=0.001

At $1.6mg/cm^2$ mean CPT for 20% DEET= 445 mins (Std =5) and PMD-XL = 635 (Std 5), p= 0.001

ED_{95} and half-life

ED_{95} estimated from Probit analysis, and half-life ($t_{1/2}$) values are shown in Table 1. 20% DEET (1) are the results when DEET was compared to PMD and 20% DEET (2) when compared to PMD-XL.

Half-life ($t_{1/2}$) values in hr. were calculated using a non-linear regression analysis from the formula derived using XLSTAT, i.e. Repellence = pr3/(1+Exp(-pr1+pr2*Time)) where pr1, pr2 and pr3 are constants, and $t_{1/2}$ = pr1/pr2. The following equations were obtained:

Protection = 1/(1+Exp(-4.9 -+1.8*Time)) … DEET (1) (R^2=0.92)

Protection = 1.01/(1+Exp(-7.0 +2.5*Time)) … DEET (2) (R^2=0.94)

Protection = 1.36/(1+Exp(-0.97 + 0.44*Time)) … PMD ($R^2= 0.91$)

Protection = 1/(1+Exp(-5.5 +1.4*Time)) … PMD-XL ($R^2= 0.84$)

DEET (1) refers to the first experiment when comparing to the 30% PMD treatment, and DEET (2) that when repeated to compare with the PMD-XL option.

Discussion

In the study comparing 20% DEET to 30% PMD in ethanol, the dose/complete protection time is in agreement with the logarithmic relationship first described by Beuscher et al[2] for *Aedes ae*. Their derived constants for the regression equation differ from those in this study, which is not unexpected since the conditions were quite different from those of modern cage tests. The regression curve can be used to compare protection times to other cage tests of a similar design for other applied doses, although as reported by Barnard et al.[6], these can vary somewhat depending on test conditions related to the number of mosquitoes, cage size, and timing of testing. For example, in our study, a protection time of 5.5 hr. for 0.25 mg/cm^2 active ingredient (ai) and 8 hr. at 0.4 mg/cm^2 ai DEET can be derived from the regression equation. This can be compared to other studies by Schreck[7], who reported a protection time of 4.8 hr. using 0.25 mg/cm^2, and 8.2 hr. at 0.4 mg/cm^2 ai [8]all on *Ae. aegypti*. Barnard *et al.*[6] reported 5-6 hr. at an applied dose of 0.4mg/cm^2, while Colucci and Muller[9] obtained a value of only 0.5 hr. at 0.2mg/cm^2.

The ED_{95} of DEET (0.03 mg/cm^2) is similar to that of Badolo *et al*.[10] for *Aedes aegypti*, and also to that of Klun et al.[11], but slightly higher than that of Debboun et al.[12] The half-life, which represents the rate of decline in repellence after the CPTe, is not often reported in such studies. For DEET, Constantini *et al*.[13] estimated this parameter as 2.9 hr. in a field study on *Anopholes*, a result similar to our findings, and in the original work by Rutlidge,[1] it was only 0.67 hr. using a different cage test procedure. It could be argued that assessing decline in repellence does have some value in that it would represent a continued degree of protection, which under circumstances of low biting pressure may be acceptable.

To date, there has not been a published study on the relationship between CPT and dose for PMD. Our results confirm that it does follow the same log-dose relationship for duration, with very similar estimated coefficient constants for the non-linear regression statistics of responses to DEET. The fit to the model is very good for both DEET and PMD, with R^2 values >90%. However, there are not many reports available for PMD using *Ae. aeygpti* with which to compare these results. Shrek[5] noted just one hr. protection at 0.5 mg/cm^2, compared to our derived CPT of 6.4 hr. The study by Carroll and Loye[4] gave a more varied response from their subjects, with PMD at a dose of 0.3 mg/cm^2 having a maximal effect of as much as 5 hr., as in our study. However, in 2 out of 8 subjects, this value was only 2 hr.; this contrasts with a measure of 8 hr. for DEET at 0.5 mg/cm^2.

The ED_{95} for PMD was lower than that reported by Trigg and Hill[5] for *Ae. aegypt*i (0.108 v 0.3mg/cm^2), although this does confirm the lower dose-equivalent potency of PMD when compared to DEET. They also measured the decline in protection of PMD and compared this measure to that obtained with DEET, and although they did not report a figure for a half-life, from their data there is only a small difference between the two repellents, an observation noted in our study.

Vanillin has previously been reported to prolong the protection time of DEET.[14] The longer-acting PMD product (PMD-XL) also contains vanillin, an aromatic aldehyde which forms an PMD-aldehyde adduct(s) when combined with 30% PMD in this formulation. Indeed, the longevity effect observed for this formulation presumably arises from the generation of PMD-vanillin hemiacetal, and more likely acetal species, via reversible chemical reactions; these agents may indeed serve as latent sources of the active PMD ingredient. This has been shown by Carroll et al. to result in a repellent having a CPT which is greater than double that of 25% DEET when applied at a dose level of 1.6 mg/cm^2 in their cage test. In our tests examining the relationship between CPT and applied dose, an

S-shaped sigmoidal relationship was observed for PMD-XL. This has important implications when comparing the activity of this product to DEET formulations, since when applied at a dose of 1.6 mg (Figure 2), our results confirm those of Carroll et al. [15] that a much longer CPT than that of DEET results, in this case approximately 1.5-fold the length of protection. However, at lower doses of 0.8-1 mg/cm^2, less advantage is offered.

It has been shown that travellers to endemic areas for mosquito-borne diseases tend to apply on average only 1mg/cm^2 of a repellent to their exposed arms rather than the higher doses at which cage tests are performed in line with the WHO protocols[16,17]. The ED_{95} for PMD-XL was similar to, but the half-life following CPT application was substantially longer than that of PMD alone. At this point, the unusual dose relationship observed for PMD-XL is not simply explicable. This requires further investigation, and more importantly, the identification of such features also exhibited by other longer-acting formulations of repellents such as those employing microcapsules or complex polymers. It should also be noted that in this second assessment, the CPT of DEET at all doses is somewhat lower than when assessed in the first study design, despite using the same conditions and subjects, and this indicates the degree of variability associated with this particular measure.

We recommend that assessing a full dose-response curve for a repellent formulation against DEET would better facilitate comparisons of the activities of repellent products. For example, it can be calculated from our regression curve that 20% DEET applied at 1.6 mg/cm^2 gives a CPT of 323 minutes (5.4 hours). However, 50% (w/v) DEET products are also available, so if this was applied at the same dose, then from our analysis, the CPT value would be as much as 364 minutes (6 hours).

Authors Contributions

LG conceived the study; LG, KD and MP designed the study protocol; KD and MP carried out the cage tests; LG and MG carried out the analysis and interpretation of data. LG, MD and MG drafted the manuscript. All authors read and approved the final manuscript. LG and MG are guarantors of the paper.

Acknowledgements

This paper is dedicated in memory of Dr John Hywel Davies, founder and CEO of Neo-Innova who passed away in October 2019. His personal support and advice made this work possible, and he will be greatly missed by all his colleagues working in the area of repellent research.

Funding

This work was supported by Neo-Innova who developed PMD-XL

Ethical Approval

Ethics approval for this study was obtained from the Rao Nursing Home, Pune India Ethics Committee (ECR/597/Inst/MH/2014/RR-17).

Figure 1. Complete Protection times with increasing applied dose of 20 % DEET and 30% PMD based on nonlinear Regression equations.

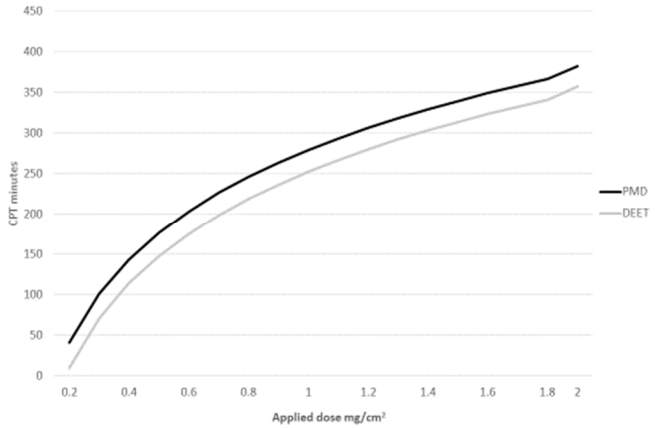

Figure 2. Complete Protection times for increasing applied dose of 20% DEET and 30% PMD-Xl based on non-linear regression equations.

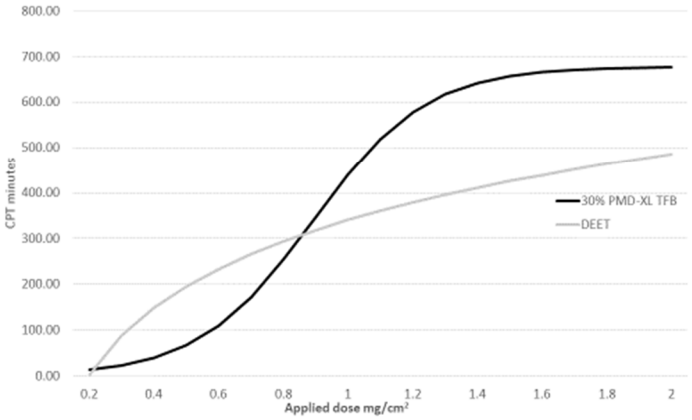

Table 1. ED_{95} and Half lives of Repellents

	30% PMD	30% PMD-XL	20% DEET (1)	20% DEET (2)
ED_{95} (95% CI) mg/cm^2	0.25(0.23,0.26) *(0.18 ai)**	0.24 (0.23, 0.26) (0.072 ai)*	0.09 (0.08, 0.098) (0.0286 ai)*	0.086 (0.078, 0.095) (0.0286 ai)*
Half life (95% CI) hours	2.23 (2.14, 2.32)	3.8 (3.65,3.95)	2.74 (2.66,2.82)	2.87 (2.80, 2.94)

**Equivalent value of ED_{95} if using 100% of active ingredient*

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