DRUG ANALYTICAL RESEARCH
ISSN: 2527-2616 *www.seer.ufrgs.br/dar*

Evaluation of quality and safety parameters of DEET commercial repellents: photostability, penetration/permeation and eye irritation studies

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N',N'-diethyl-m-toluamide (DEET) is the repellent most commonly used against mosquito vectors of diseases such as dengue, yellow fever and chikungunya. In Brazil, DEET is marketed in aerosol, solution, lotion and gel forms, at concentrations ranging from 6.65 to 25%. In this study, the degradation kinetic under UVC radiation was studied, as well as the penetration/permeation and the ocular irritant potential of DEET commercial repellents in the form of solution, lotion and gel. The photostability study was conducted over 96h, and the DEET degradation kinetics under UVC radiation was fitted to the zero-order model for the three formulations; t90% values of 23.7 h, 17.0 h and 16.1 h were obtained for gel, lotion and solution forms, respectively. The in vitro skin penetration/permeation in pig skin using the vertical Franz cell showed that all the formulations penetrated/permeated the skin layers, at a higher rate when the lotion ($p<0.05$) was used, possibly due to its qualitative composition. The in vitro ocular irritant potential using the HET-CAM method indicated that one of the products evaluated was classified as "moderate irritant" and five as "severe irritant". The set of data indicated the lower penetration/permeation of the solution form and reinforces the importance of being careful during application, to avoid accidental contact with the eyes.

Keywords: Penetration/permeation; HET-CAM; degradation kinetics; repellents; DEET.

Article received at 06/01/2022 and accepted at 07/11/2022.
https://doi.org/10.22456/2527-2616.124882

Introduction

Severe human afflictions like dengue, yellow fever and chikungunya are transmitted by arthropod vectors like mosquitoes [1]. Recent data from the Brazilian government on arbovirus cases in the country indicate that, in the first sixteen weeks of 2022, 47,281 probable cases of chikungunya and 2118 probable cases of zika were reported, which represent a 40% and 54% increase compared to the same period in 2021, respectively [2].
The scenario of dengue is the worst with 542,038 probable cases of dengue (incidence rate of 254.1 cases per 100 thousand inhabitants), which represents a 113.7% increase of cases recorded for the same period in 2021. The incidence rate in the South Region is the second highest in the country, with 427.2 cases per 100 thousand inhabitants [2]. Large dengue epidemics have a very peculiar characteristic: they occur with a certain frequency, with an interval of two to three years between them. This periodicity is related to a combination of climatic conditions that favor the multiplication of *Aedes aegypti* and the collective immunity resulting from the previous circulation of the four serotypes of the virus. Therefore, periodically the impact of dengue is different in the states [3]. Other factors related to the increase include the concomitance with the COVID-19 pandemic and the epidemic of H3N2 influenza, which limited resources for disease prevention, the growing number of people with housing problems and the precariousness of cities, making it difficult to control vectors that transmit these diseases [4].
When infestation by mosquitoes occurs, the control is carried out using appropriate clothing, minimizing skin exposure or using insecticides and insect repellents, since a vaccine is available in the public health system only for yellow fever [5]. In this scenario, *N',N'*-diethyl-*m*-toluamide (DEET) is the main repellent used against mosquito vectors of these diseases [6]. In Brazil, DEET is found on the market in aerosol, solution, lotion and gel forms, at concentrations ranging from 6.65 to 25%.
Preliminary results obtained by our research group showed that DEET is degraded under UVC radiation [7]. Exposure of drugs and cosmetic active ingredients to visible and UV radiation is inevitable, especially after topical application of the product. Thus, products that are photosensitive can degrade, rendering the topical application of many substances ineffective [8,9]. Besides, the photostability study is currently an important tool to suggest the stability of drugs and pharmaceuticals in industry [10].
The active product ingredient of the repellents can also cross biological barriers, reach the systemic circulation and then cause damage [11]. The subchronic dermal application of DEET in adult rats resulted in the death of diffuse neuronal cells in the cerebral cortex, hippocampus and cerebellum, leading to many physiological, pharmacological and behavioral abnormalities, particularly memory dysfunction and motor and learning deficits [12]. Studies on DEET penetration/permeation evidenced that it presents high skin permeation leading to local and systemic effects, especially with products that contains ethanol as solvent [13], or by the concomitant

application of sunscreens, due to synergistic percutaneous permeation of the insect repellent DEET and the sunscreen oxybenzone [14,15]. However, to the author's best knowledge, comparative studies on DEET permeation with different formulations have not been reported in the literature.

Besides the potential problems caused by skin permeation, repellents can accidentally reach the eyes and cause irritation. Macrae et al. described the ocular irritation potential of DEET in bulk by using the Draize ocular test [16]. Alternatives to *in vivo* tests have been described and the hen's egg chorioallantoic membrane test (HET-CAM) has been widely disseminated as a valid model replacing the Draize ocular test.

In this scenario, our study aimed to determine the kinetic degradation, the skin permeation/penetration and to evaluate the ocular irritation potential by the HET-CAM method of DEET-based commercial repellents, comparatively between the different available pharmaceutical forms.

Experimental

Chemicals and reagents

DEET standard (98.8%) was obtained from Merck-Sigma-Aldrich (Darmstadt, Germany). Methanol, acetonitrile and ethanol (HPLC grade) were purchased from Merk-Sigma-Aldrich (Darmstadt, Germany), Sigma-Aldrich (St Louis, USA) and Panreac (Barcelona, Spain), respectively. Ultrapure water was obtained from a Megapurity Water Purification System. Phosphoric acid, used to adjust the mobile phase pH, was purchased from Tedia (Fairfield, USA).

The following commercial repellents were used in this study: Repelex® lotion, Repelex® gel Kids, Repelex® solution (Reckitt Benckiser, Berkshire, United Kingdom), OFF® solution, OFF® refresh solution (Johnson & Son, Ontario, Canada), Affast solution (Cimed, São Paulo, Brazil). All of them were acquired in the local market and were named as F1 to F6. Repellents F1, F2 and F3 were used in the photodegradation and permeation/penetration studies (for qualitative composition see Table 1).

All six products were used in the *in vitro* ocular irritation test. F4, F5 and F6 were similar to F3, i.e., DEET alcoholic solutions.

Chromatographic system

A Shimadzu LC-System (Kyoto, Japan) equipped with an LC-20AT pump, SPD-M20A detector (DAD detector), DGU-20A5 degasser, CBM-20A system controller, SIL-20A HT auto sampler and LC-Solution software was used. As stationary phase an RP-18-column (Inertsil ODS-3, 150 mm x 4.6 mm; 5µm) (Tokyo, Japan) was employed and as mobile phase a mixture of methanol, acetonitrile and acidic water (pH 4.5) (45:10:45), at a flow rate of 1.0 mL min^{-1}. The pH of water was adjusted with phosphoric acid, the injection volume was 50 µL and the detection was at 270 nm. The method was previously validated according to ICH guidelines, and was appropriate to perform stability studies of different DEET products [7].

Table 1. Qualitative composition of formulations used in the photodegradation and permeation/penetration studies.

Formulation	Qualitative composition
F1 (lotion)	DEET 6.79%; cyclomethicone; PPG-6-C12-15; aloe barbadensis leaf powder; cetearyl alcohol; sodium hydroxide; water; phenoxyethanol; methylparaben; ethylparaben; propylparaben; petrolatum; parfum; amyl cinnamal; benzyl benzoate; cinnamyl alcohol; citronellol; limonene; eugenol; geraniol; hexyl cinnamal; hydroxyisohexyl 3-cyclohexene carboxaldehyde; isoeugenol; linalool
F2 (gel)	DEET 7.34%; alcohol; hydroxypropyl cellulose; parfum; aqua; linalool; geraniol; limonene
F3 (solution)	DEET 6.79%; alcohol; isopropyl myristate; parfum; isoeugenol; linalool; amyl cinnamal; benzyl benzoate; cinnamyl alcohol; citronellol; limonene; eugenol; hexyl cinnamal; hydroxyisohexyl-3-cyclohexene-carboxaldehyde

Photodegradation kinetics

Photodegradation kinetics of F1, F2 and F3 was studied under UVC light (254 nm). Sample solutions were prepared at the concentration of 1000 µg mL^{-1} as following: at first the semisolids (lotion and gel) densities were measured with a pycnometer, and then a quantity equivalent to 25 mg of DEET was weighed in a 25 mL volumetric flask. Fifteen milliliters of ethanol were added and after 5 minutes of stirring, the volume was made up. To prepare the DEET solution, a volume corresponding to 25 mg of DEET was transferred to a 25 mL volumetric flask and ethanol was added to complete the volume. Then, 1 mL of each solution was transferred to a quartz cell and exposed to UVC light throughout a 96-hour period in a mirrored light chamber. At time intervals of 0, 12, 24, 48, 72 and 96 hours the samples were diluted to a theoretical concentration of 50 µg mL^{-1} and analyzed. Samples protected from light were exposed to the same conditions to evaluate the interference of heat inside the chamber.

The graphic method was used to determine the order of reaction. A correlation coefficient (r) closer to one was chosen to describe the model. Parameters such as degradation rate constant (k), t50% and t90% were calculated [17].

In vitro skin penetration/permeation

An in vitro skin penetration/permeation study was performed using Franz-type diffusion cells (3.14 cm^2 diffusion surface and 9.0 mL receptor volume), using the formulations F1, F2 and F3. Fresh abdominal pig skin was obtained from a local abattoir (Erechim, Brazil) and

used as a membrane, because of its morphology and permeability similar to human skin [18]. Before the study, the visible hairs were shaved and the subcutaneous fat was removed. Pieces of skin were cut in circles and were stored at -18 ºC until the time of use. The receptor medium was composed of phosphate buffer pH 7.4 containing 0.5% polysorbate 80, which was chosen to ensure the DEET sink condition. About 70 mg (corresponding to an infinite dose of each formulation (F1, lotion; F2, gel and F3, solution) was applied to the skin surface. During 8 hours, the cells (n= 4/formulation) were maintained in a thermostatic bath at 32 ± 0.5°C, with continuous stirring of the receptor fluid at 200 rpm. At the end of this period, the cells were disassembled, the formulation excess was removed with cotton wool and the DEET distribution in the skin was investigated.

Stratum corneum was removed by tape stripping using 18 successive tapes (Scotch tape 3M, USA). Epidermis and dermis were separated by heating the skin in a water bath (60 ºC) during 60 s. DEET was extracted from each skin layer with the mobile phase, followed by vortex mixing during 2 min and sonication (15 min). In addition, aliquots of the receptor medium were collected and assayed for DEET content, as well as the cotton wool that was used to remove the residual formulation over the skin. The samples were assayed by the HPLC method already described. The method was previously revalidated, being linear in the range of 0.5–50 µg mL^{-1}. Recovery (%) was evaluated by the analyses of the amount of DEET assayed in full skin samples previously contaminated with a known amount of DEET in solution (0.5, 5.0 and 50.0 µg mL^{-1}) and subjected to the same experiment described above. High drug recovery was achieved from the full pig skin (96.14 ± 0.48%). Furthermore, specificity was tested for all sample matrices (tape strip, viable dermis and dermis) and no interference of components of these matrices in the chromatographic peak of DEET was observed.

In vitro ocular irritation test

HET-CAM was chosen to study ocular irritation *in vitro* and the protocol followed the recommendations of INVITTOX [19]. For the test, fertile chicken eggs incubated per 10 days, kept at 38 ± 0.5°C and relative humidity 70%, were obtained from a local poultry farm (Teutônia, Brazil). The eggshell around the air chamber region was removed with the help of a fine forceps. Carefully, the inner membrane was removed exposing the chorioallantoic membrane (CAM). Then, 0.3 mL of each formulation was applied over the CAM (n= 4/formulation). After 20 seconds the membrane was washed with sodium chloride (NaCl) 0.9% and reactions such as hemorrhage, vasoconstriction, and coagulation were observed during 300 seconds. The time to the onset of each reaction was monitored and recorded in seconds. NaCl 0.9% and sodium hydroxide (NaOH) 0.1M were used as negative and positive control, respectively. The irritation score (IS) was calculated according to the following equation, where h = hemorrhage time; v = vasoconstriction time, and c = coagulation time.

$$IS = [5 (301 – h)/300] + [7 (301 – v)] + [9 (301 – c)/300]$$

According to the IS values, the formulations were classified as non irritant (0-0.9), slight irritant (1-4.9), moderate irritant (5-8.9) and severe irritant (9-21). Formulations F1 to F6 were evaluated by this test.

Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA), post hoc Tukey's test at a significance level of 5%.

Results and Discussion

UVC photodegradation kinetics

In our previous forced degradation tests, UVC radiation provided the highest DEET degradation value among the conditions used. Although most UVC radiation is filtered by ozone in the upper atmosphere, the study of the chemical and biological effects of this radiation has been increasing not only to improve knowledge of the photochemistry of this radiation but also because of the damage it causes [20]. In addition, the route of DEET administration supports the relevance of this evaluation.

According to ICH guidelines [21], the photostability test of dermal products should be performed to support their photostability in-use and the extent of this testing should depend on and be related to the directions for use, and is left to the applicant's discretion. Liquid samples may have a high optical density, thus leading to a severe filter effect, i.e., only a thin surface layer can absorb the radiation, thereby protecting the inner part of the preparation from exposure. The filter effect should be avoided in kinetic and mechanistic studies that can be obtained by diluting and stirring the samples [22]. Thus, the DEET products were diluted before the exposure to UVC radiation.

During 96 hours samples were analyzed at times 0, 12, 24, 48, 72 and 96 h (Figure 1). This time was necessary to obtain a relevant degradation that is important to establish an unequivocal delineation of the reaction order [22].

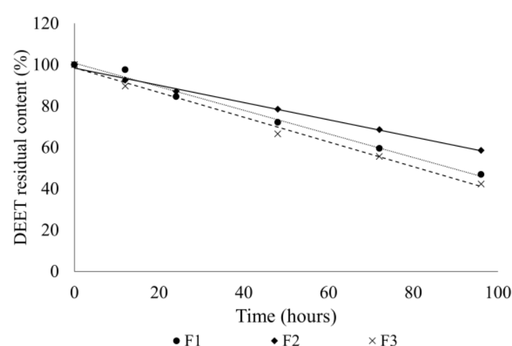

Figure 1. DEET degradation profile in UVC kinetics study at times 0, 12, 24, 48, 72 and 96 hours, for the lotion (F1), gel (F2) and solution (F3).

The degradation kinetic of the three analyzed formulations was determined to define the reaction order (zero, first, or second order) and the reaction rate [17]. Analysis of the correlation coefficients that were obtained by plotting the drug concentration versus time, log of drug concentration versus time and the inverse of concentration versus time indicated that all the formulations were degraded following zero order kinetics, with a linear relation between concentration versus time, represented by equation $C = C0 - kt$. The slope of the straight line indicated the reaction constant (k) and expressed the fraction of drug that reacts per time; the t90% and t50% were estimated from the k value, and can be observed in Table 2. These data indicated the following degradation order, F3 > F1 > F2.

This result could be related to the excipients of each formulation, which can have different effects. Cosolvents and surfactants can have a photo-stabilizing or -destabilizing effect on the product that may be assigned to a change in sample absorbance due to an increase in solubility and dissolution of particle aggregates or to a change in the polarity and in some cases the viscosity of the medium [23]. In addition, substances with absorption spectra similar to that of the drug can protect it from photodegradation, which is known as the overlay spectral principle [23].

Although degradation was observed, the t90% value for the three formulations was higher than the product reapplication intervals, which range from 2 h to 8 h, even under UVC radiation. Therefore, this result will not compromise the efficacy of the repellent. Additionally, in a previous study using UVA radiation, degradation of 2.8%, 1.21% and 0.64% (F3, F1 and F2, respectively) was observed after exposure to an 800 Wh m^{-2} radiation level [7], that can be equivalent to 4 to 8 days on a sunny window sill [22]. These sets of results indicate the photostability of DEET formulations.

Table 2. Kinetic data of DEET degradation in different formulations.

Product	k	$t_{50\%}$ (h)	$t_{90\%}$ (h)
F1	0.5887 ± 0.005^{a}	85.06 ± 0.65^{ab}	17.01 ± 0.13^{ab}
F2	0.4346 ± 0.051^{b}	118.62 ± 13.49^{a}	23.73 ± 2.70^{a}
F3	0.6214 ± 0.011^{a}	80.46 ± 1.45^{b}	16.09 ± 0.29^{b}

Results expressed as mean ± SD (n = 2). At the same column, values followed by the same letter do not differ (One-way ANOVA, followed by Tukey *post-hoc* test, α=0.05).

In vitro skin penetration/permeation

The penetration/permeation of substances into the skin depends on the physico-chemical characteristics of the substance, the inherent characteristics of the formulation and also on the physiological conditions of the application site [24]. DEET has some characteristics that facilitate its permeation, such as low molecular weight (MW = 191.3 Da) and moderate lipophilicity (log P = 2.5) [25].

To determine the drug localization and to quantify the DEET delivered to each skin layer after applying three different formulations (lotion, gel and solution), an *in vitro* study was performed using vertical Franz diffusion cells and porcine skin as biological membrane. DEET was analyzed in the stratum corneum, by the tape stripping technique, and in the epidermis and dermis, using skin extraction techniques.

Concerning distribution through the skin, F1 provided homogeneous DEET levels through the skin layers, with no difference between its concentrations in stratum corneum, epidermis and dermis (p>0.05). F2 and F3 showed higher levels of DEET retained in the stratum corneum when compared to the other skin layers ($p < 0.05$) (Figure 2). The receptor compartment was also analyzed and DEET was detected demonstrating that the drug was able to permeate the skin using all three formulations. The following values were found: F1, 148.2 µg/cm^2; F2, 67.17 µg/cm^2 and F3, 7.08 µg/cm^2, that are in consonance with the values found in the dermis. The lower drug level found in the dermis suggests that lower DEET systemic absorption can be expected from the F3.

Additionally, the cotton wool used to remove the remaining formulations on the skin at the end of 8 hours was also analyzed. As expected, a lower DEET level was found in the cotton wool used to remove the formulation with the higher permeation value: F1 4934 µg mL^{-1}, F2 24087 µg mL^{-1} and F3 45436 µg mL^{-1}.

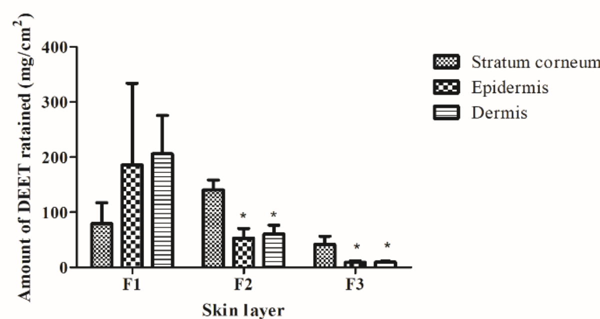

Figure 2. Amount of DEET retained in stratum corneum, epidermis and dermis after 8 hours from DEET in lotion (F1), gel (F2) and solution (F3). The results are expressed as mean ± standard error (n = 4). For the same formulation *indicated a significant difference from the value retained in the stratum corneum. Statistical analysis was performed using analysis of variance (ANOVA) and Tukey's post-hoc test at 5% of significance, with the help of statistical software RStudio® (Massachusetts, USA).

Increasing skin hydration in general, may increase the transdermal delivery of hydrophilic and lipophilic substances. This can be achieved by tissue occlusion, to avoid transepidermal water loss, or by exposing the skin to high humidity [26]. In this context, differences in the qualitative composition of the formulations studied could explain the differences in the permeation of DEET. F2 and the F3 contain alcohol as excipient, a recognized absorption promoter and high values could be expected. However, F1 contains emollients, such as petrolatum, cyclomethicone and cetearyl alcohol that can occlude the skin, helping in the permeation. Terpenes like limonene and eugenol, other components of F1, are also described as skin penetration enhancers [26]. Considering that the

dermis is the first vascularized skin layer, substances that reach this layer will be available in the circulation and may cause systemic effects [26].
Our results are in accordance with reported studies performed with DEET in solution [27] or in combination with oxybenzone sunscreen [15,28,29], that also demonstrated the DEET penetration/permeation potential.

In vitro ocular irritation test

The ocular irritant potential of the studied formulations was evaluated by the HET-CAM method. Positive control (NaOH 0.1M) was classified as "severe irritant" and negative control (NaCl 0.9%) did not cause any reaction. Of the six samples analyzed in our study, five were considered as "severe irritant" and one as "moderate irritant" (Table 3).
Using the conventional Draize method, DEET presented an irritant potential when tested in the presence of different concentrations of ethanol (100% ethanol, 100% DEET and EtOH: DEET (80:20) and also when considering the amount applied (10, 30 or 100 µL) [16]. The results found in our study corroborate those previously published, carried out only with ethanolic solutions of DEET, where the products showed ocular toxicity.

Table 3. Ocular irritation rate and consequent classification of studied formulation by HET-CAM method.

Repellent	Ocular irritation rate*	Classification
F1 (lotion)	8.04 ± 0.35	Moderate irritant
F2 (gel)	10.68 ± 0.31	Severe irritant
F3 (solution)	14.94 ± 1.03	Severe irritant
F4 (solution)	11.04 ± 1.25	Severe irritant
F5 (solution)	9.75 ± 0.40	Severe irritant
F6 (solution)	9.50 ± 1.51	Severe irritant

*The results are expressed as mean ± SD (n = 4).

Conclusion

The DEET degradation kinetics under UVC radiation was fitted to the zero-order model according to the degradation profiles, for the three formulations. Although degradation occurred, this result does not compromise the efficacy of products studied, considering the repellent posology and the radiation source used. The in vitro skin penetration/permeation study demonstrated the permeability of DEET across skin layers, with lower penetration/permeation observed using the solution form. DEET also exhibited ocular irritation potential regardless of the type of formulation applied, which emphasizes the need for care during application.

Acknowledgments

The authors wish to thank FIPE Senior (UFSM) and CAPES for the financial support.

Conflict of interest

The authors declare no conflict of interest.

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