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EVALUATION OF AN IN VITRO BLOODFEEDING SYSTEM FOR TESTING MOSQUITO REPELLENTS[1]

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ABSTRACT. Median effective doses and 95% effective doses of 9 commercial mosquito repellents were determined for the yellow fever mosquito, *Aedes aegypti,* in an in vitro bloodfeeding test system and on the human forearm. Results obtained in the 2 test systems did not differ significantly but, because of the inherent variability of repellent test data, did not always agree closely. Potential modifications of in vitro bloodfeeding test systems for increased accuracy, precision, and reliability are discussed.

KEY WORDS Mosquito repellents, *Aedes aegypti,* mosquito, repellents

INTRODUCTION

Bar-Zeev and Smith (1959) introduced the use of in vitro bloodfeeding systems for testing mosquito repellents with a system that used 9 glass feeders at which mosquitoes could feed through Silverlight™ membranes on citrated animal blood from below. Effective doses of deet, dimethyl phthalate, and ethyl hexanediol were determined by treating the membranes with graded doses of the test materials. A separate cage of female mosquitoes (*Aedes aegypti* L.) was placed at each feeder, with the system functioning as a no-choice test system.

Subsequently, Rutledge et al. (1976) introduced an in vitro bloodfeeding test system that used 5 acrylic plastic or glass feeders at which mosquitoes could feed through goldbeater's skin[4] on heparinized human blood from above. Effective doses of deet were determined by treating the membranes with graded doses of the test material. A single cage of female mosquitoes (*Ae. aegypti*) was positioned to allow feeding at any feeder ad libitum, with the system functioning as a free-choice test system.

Median effective doses (ED_{50}s) and 95% effective doses (ED_{95}s) of various commercial and experimental repellents for *Anopheles stephensi* Liston, *An. albimanus* Wied., *An. quadrimaculatus* Say, *Culex pipiens* L., *Cx. tarsalis* Coq., *Ae. aegypti,* and *Ochlerotatus taeniorhynchus* (Wied.) were subsequently determined (Rutledge et al. 1978, 1983; Skinner et al. 1979a, 1979b, 1980; Reifenrath and Rutledge 1983).

To date, no comparison of results obtained in tests with an in vitro bloodfeeding test system with results obtained in comparable tests on humans has been published. The purpose of the present study was to compare results obtained in tests with the in vitro bloodfeeding test system of Rutledge et al. (1976) with results obtained in comparable tests (American Society for Testing and Materials 1983) on humans and to evaluate and analyze the in vitro error. The research was conducted at the former Letterman Army Institute of Research, Presidio of San Francisco, CA, over the period 1975–79.

MATERIALS AND METHODS

Test species: The mosquitoes used in the study were 5- to 15-day-old nulliparous female *Ae. aegypti* (University of California at San Francisco strain). The colony was maintained as described by Rutledge et al. (1978).

Test materials: Materials tested were deet (*N,N*-diethyl-3-methylbenzamide), ethyl hexanediol (2-ethyl-1,3-hexanediol), dimethyl phthalate (dimethyl phthalate), butopyronoxyl (butyl 3,4-dihydro-2,2-dimethyl-4-oxo-2*H*-pyran-6-carboxylate), Citronyl® (S. C. Johnson and Son, Inc., Racine, WI), (3-acetyl-2-(2,6-dimethyl-5-heptenyl)-oxazolidine), dibutyl phthalate (di-*n*-butyl phthalate), butoxypolypropylene glycol (butoxypropanediol polymer), MGK Repellent 11® (McLaughlin, Gormley King Corp., Minneapolis, MN) (1,5a,6,9,9a,9b-hexahydro-4a(4*H*)-dibenzofurancarboxaldehyde), and MGK Repellent 326® (McLaughlin Gormley King Corp.) (di-*n*-propyl-2,5-pyridinedicarboxylate).

All test materials were technical grade, obtained from commercial sources. None, except dimethyl phthalate and dibutyl phthalate, were related in chemical structure. All are or have been used in commercial repellent formulations in the United States.

In vitro test procedure: The 5-feeder configuration of the in vitro bloodfeeding test system was used (Rutledge et al. 1976). The 5 membranes were treated at random with a control (ethanol) and 4 serial dilutions (initially, 0.02, 0.04, 0.08, and 0.16

[1] Opinions and assertions herein should not be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Use of trade names does not imply official endorsement or approval of the products named.

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[4] Goldbeater's skin is the prepared outside membrane of the large intestine of the ox, which is used by goldbeaters to separate leaves of gold foil when gold is beaten. In the entomological literature it is often called Baudruche membrane, from the French *baudruche,* meaning goldbeater's skin (De Vries 1976). According to Tarshis (1958), the Silverlight membrane of Bar-Zeev and Smith (1959) was a brand of goldbeater's skin.

mg/cm^2) of the test material in ethanol. A 30 × 30 × 30-cm acrylic plastic test cage containing 250 female mosquitoes was placed over the feeders, and a slide in the bottom was withdrawn to allow the mosquitoes access to the membranes. The number of mosquitoes feeding on each membrane was recorded every 2 min for 20 min, and the totals of the 10 counts of feeding mosquitoes on each membrane were obtained by addition. In tests of this type against *Ae. aegypti,* the total obtained for the control membrane is typically about 150.

Repellents were tested at 1 or more successive ranges of dose as needed to determine a test range that bracketed the ED_{50} and ED_{95}. Each repellent was then tested 4 times at that range of doses, except for deet (6 times). The totals for each dose of each repellent and the corresponding controls were obtained on completion of testing. Dose totals were converted to percent of the corresponding control total and subtracted from 100% to express the response to the test material in terms of the percent of mosquitoes repelled.

Forearm test procedure: Forearm tests were conducted in accordance with Standard E951-83 of the American Society for Testing and Materials (1983). Test subjects gave free and informed consent, and the investigators complied with applicable laws and regulations on the use of human subjects in research.

Five 29-mm-diameter circular test areas were outlined on the flexor surface of the forearm and treated at random with a control (ethanol) and 4 serial dilutions (initially, 0.002, 0.004, 0.008, and 0.016 mg/cm^2) of the test material in ethanol. A 4 × 5 × 18-cm acrylic plastic test cage containing 15 mosquitoes was secured to the forearm, and a slide was withdrawn to allow the mosquitoes access to the test areas through matching 29-mm-diameter holes in the floor of the cage. The number of mosquitoes feeding on each test area was recorded at the end of 90 sec. In these tests, the average number of mosquitoes feeding on the control area was 3.8.

Repellents were tested at 1 or more successive ranges of dose as needed to determine a test range that bracketed the ED_{50} and ED_{95}. Multiple, overlapping test ranges of dibutyl phthalate (2 test ranges) and MGK Repellent 326 (4 test ranges) were tested and analyzed separately to provide multiple, independent estimates of the ED_{50}s and ED_{95}s of those repellents. Repellents were tested 12 times at each range of doses selected, except for dimethyl phthalate (28 times), Citronyl (14 times), dibutyl phthalate (8 and 10 times), and MGK Repellent 326 (4, 12, 19, and 10 times). The totals for each dose of each repellent and the corresponding controls were obtained on completion of testing. Dose totals were converted to percent of the corresponding control total and subtracted from 100% to express the response to the test material in terms of the percent of mosquitoes repelled.

Dose–response analyses: Doses (mg/cm^2) and responses (%) were converted to logarithms and probits, respectively, for analysis. The ED_{50}s and ED_{95}s and their associated confidence limits were computed for each test material at each test range by the method of Goldstein (1964) for graded responses.

In vitro error: In metrology, error is defined as "the difference between the measured value and the true value" (Busch 1989). In the present study, the value of the ED_{50} or ED_{95} obtained in vitro is analogous to the measured value of metrology, and the corresponding value obtained on the forearm is analogous to the true value of metrology. For purposes of the present study, then, error or in vitro error is defined as the difference between the value obtained in vitro and the corresponding value obtained on the forearm. However, in interpreting the results presented it should be remembered that the value obtained on the forearm is not a fixed or true value as in metrology, but a statistic that is itself variable and contributes to the observed error.

RESULTS

Dose–response analyses

Table 1 shows the ED_{50}s and ED_{95}s obtained in the in vitro and forearm tests with their respective confidence limits. Values of the ED_{50} ranged from 0.003 mg/cm^2 (MGK Repellent 326) to 0.113 mg/cm^2 (ethyl hexanediol) in the in vitro tests and from 0.004 mg/cm^2 (deet, dimethyl phthalate, butopyronoxyl, and MGK Repellent 11) to 0.385 mg/cm^2 (dibutyl phthalate) in the forearm tests. Values of the ED_{95} ranged from 0.040 mg/cm^2 (MGK Repellent 11) to 0.671 mg/cm^2 (ethyl hexanediol) in the in vitro tests and from 0.013 mg/cm^2 (deet and dimethyl phthalate) to 1.272 mg/cm^2 (dibutyl phthalate) in the forearm tests.

In several cases, the upper confidence limit of the ED_{95} was computed to be in excess of 2 mg/cm^2. Because runoff doses of liquid repellents are approximately 2 mg/cm^2 (Rutledge 1988), estimates in excess of that amount are purely statistical and are omitted from Table 1, as indicated by the ellipses (. . .).

In vitro error

Table 2 shows the ED_{50}s and ED_{95}s obtained in the in vitro and forearm tests and the corresponding estimates of in vitro error obtained by subtraction of the value obtained on the forearm from that obtained in vitro. Estimates of in vitro error of ED_{50}s ranged from −0.295 mg/cm^2 (butoxypolypropylene glycol) to 0.108 mg/cm^2 (ethyl hexanediol), with a mean of −0.039 mg/cm^2. Estimates of in vitro error of ED_{95}s ranged from −0.756 mg/cm^2 (butoxypolypropylene glycol) to 0.654 mg/cm^2 (ethyl hexanediol), with a mean of −0.026 mg/cm^2.

Because of the magnitude of several estimates of in vitro error, most notably those pertaining to bu-

Table 1. Effective doses (mg/cm²) and associated confidence intervals of 9 mosquito repellents tested against *Aedes aegypti* in vitro and on the forearm.[1]

Test material	In vitro test	Forearm test
	Median effective dose (ED_{50})	
Deet	0.031 (0.020–0.041)	0.004 (0.003–0.005)
Ethyl hexanediol	0.113 (0.081–0.197)	0.005 (0.003–0.009)
Dimethyl phthalate	0.066 (0.026–0.092)	0.004 (0.002–0.006)
Butopyronoxyl	0.032 (0.014–0.044)	0.004 (0.002–0.006)
Citronyl	0.004 (0.001–0.008)	0.005 (0.002–0.008)
Dibutyl phthalate[2]	0.016 (0.000–0.034)[3]	0.227 (0.113–0.877)
		0.385 (0.221–0.718)
Butoxy polypropylene glycol	0.034 (0.010–0.049)	0.329 (0.113–0.729)[3]
MGK Repellent 11	0.018 (0.004–0.024)	0.004 (0.003–0.006)
MGK Repellent 326[4]	0.003 (0.001–0.006)	0.009 (0.005–0.032)[3]
		0.025 (0.011–0.072)[3]
		0.021 (0.001–0.040)[3]
		0.034 (0.021–0.046)
	95% effective dose (ED_{95})	
Deet	0.140 (0.100–0.240)	0.013 (0.011–0.019)
Ethyl hexanediol	0.671 (0.282–. . .)	0.017 (0.009–0.114)
Dimethyl phthalate	0.206 (0.145–0.553)	0.013 (0.008–0.032)
Butopyronoxyl	0.095 (0.070–0.205)	0.016 (0.009–0.061)
Citronyl	0.059 (0.043–0.081)	0.074 (0.049–0.161)
Dibutyl phthalate[2]	0.307 (0.122–. . .)[3]	0.548 (0.277-. . .)
		1.272 (0.692–. . .)
Butoxy polypropylene glycol	0.172 (0.101–1.813)	0.928 (0.491–. . .)[3]
MGK Repellent 11	0.040 (0.030–0.200)	0.015 (0.010–0.044)
MGK Repellent 326[4]	0.060 (0.046–0.080)	0.026 (0.012–0.874)
		0.066 (0.034–. . .)[3]
		0.164 (0.076–. . .)[3]
		0.126 (0.101–0.169)

[1] Values greater than 2 mg/cm² are indicated by ellipses (. . .; see text). Level of confidence is 95% except as indicated.
[2] Two independent determinations on the forearm.
[3] 90% confidence limits.
[4] Four independent determinations on the forearm.

toxy-polypropylene glycol, the values of in vitro error were tested for outlying observations. Standardized variables corresponding to the respective values of in vitro error were computed by subtracting the mean from each value and dividing each difference by the standard deviation (Steel and Torrie 1980; Table 2). Critical values of standardized variables for Grubbs's test for outliers are given by Dunn and Clark (1974). Neither the in vitro error of the ED_{50} nor the in vitro error of the ED_{95} of butoxy-polypropylene glycol was significant in Grubbs's test for outliers ($T_1 = -1.77$, $n = 9$, $P > 0.05$ and $T_1 = -1.79$, $n = 9$, $P > 0.05$, respectively).

The coefficients of variation of the in vitro errors in ED_{50}s and ED_{95}s also were computed (Table 2). The values obtained, 57% and 56%, respectively, indicate levels of variation comparable to those of many kinds of biological data (Altman and Dittmer 1964).

As differences of paired observations, the estimates of in vitro error of Table 2 are data for the *t*-test of significance of differences between mean in vitro and mean forearm values of the ED_{50}s and the ED_{95}s (Steel and Torrie 1980). The *t*-test indicated that the results obtained in the in vitro tests did not differ significantly from those obtained in the forearm tests ($t = 0.807$, df = 8, $P > 0.4$ for ED_{50}s and $t = 0.191$, df = 8, $P > 0.8$ for ED_{95}s).

DISCUSSION

On the basis of the foregoing statistical analysis, we concluded that the data of Tables 1 and 2 do not include significant inconsistencies, irregularities, or extreme values and that, within the limits of error, the in vitro bloodfeeding test system of Rutledge et al. (1976) and the forearm test of the American Society for Testing and Materials (1983) provide equivalent results. The fact that the results do not always agree closely (Table 2) reflects the extent of the limits of error.

Although we assigned the observed error to the in vitro test system for purposes of analysis, an undetermined fraction of the observed error is attributable to the forearm test. For this reason, the values of in vitro error given in Table 2 should be regarded as liberal, or overestimated. Although it is clear from Tables 1 and 2 that greater precision, accuracy, and reliability are desirable in both the in vitro and the forearm tests, discussion will be con-

Table 2. Effective doses (mg/cm²) of 9 repellents tested against *Aedes aegypti* in vitro and on the forearm, with values of in vitro error obtained by subtraction and standardized variables corresponding to error values.

Test material	In vitro test	Forearm test	In vitro error	Standardized variable
	Median effective dose (ED_{50})			
Deet	0.031	0.004	0.027	0.46
Ethyl hexanediol	0.113	0.005	0.108	1.01
Dimethyl phthalate	0.066	0.004	0.062	0.70
Butopyronoxyl	0.032	0.004	0.028	0.46
Citronyl	0.004	0.005	−0.001	0.26
Dibutyl phthalate	0.016	0.296[1]	−0.280	−1.66
Butoxy polypropylene glycol	0.034	0.329	−0.295	−1.77
MGK Repellent 11	0.018	0.004	0.014	0.37
MGK Repellent 326	0.003	0.020[2]	−0.017	0.15
Mean			−0.039	
Standard deviation			0.145	
Coefficient of variation (%)[3]			57	
	95% effective dose (ED_{95})			
Deet	0.140	0.013	0.127	0.38
Ethyl hexanediol	0.671	0.017	0.654	1.67
Dimethyl phthalate	0.206	0.013	0.193	0.54
Citronyl	0.059	0.074	−0.015	0.03
Butopyronoxyl	0.095	0.016	0.079	0.26
Dibutyl phthalate	0.307	0.835[1]	−0.528	−1.23
Butoxy polypropylene glycol	0.172	0.928	−0.756	−1.79
MGK Repellent 11	0.040	0.015	0.025	0.12
MGK Repellent 326	0.060	0.077[2]	−0.017	0.02
Mean			−0.026	
Standard deviation			0.408	
Coefficient of variation (%)[4]			56	

[1] Geometric mean of 2 independent determinations. See Table 1.

[2] Geometric mean of 4 independent determinations. See Table 1.

[3] Because coefficients of variation are computed from positive values only, an adjusted mean, 0.256 mg/cm², was used to compute the value shown. The adjusted mean was computed as −0.039 mg/cm² (the mean) minus −0.295 mg/cm² the value of the most extreme negative observation). The effect of the adjustment is to shift the scale of values of in vitro error from the range −0.295 to 0.108 mg/cm² to the positive range 0.000 to 0.403 mg/cm². The scale shift does not affect the value of the standard deviation.

[4] Because coefficients of variation are computed from positive values only, an adjusted mean, 0.730 mg/cm², was used to compute the value shown. The adjusted mean was computed as −0.026 mg/cm² (the mean) minus −0.756 mg/cm² (the value of the most extreme negative observation). The effect of the adjustment is to shift the scale of values of in vitro error from the range −0.756 to 0.654 mg/cm² to the positive range 0.000 to 1.410 mg/cm². The scale shift does not affect the value of the standard deviation.

fined to the in vitro test, the subject of the present study.

The in vitro bloodfeeding test system was designed as a free-choice test system because it was thought that a choice test was "more comparable to the natural situation, in which the mosquito is free to seek an alternate, untreated host, or at least an untreated or thinly treated part of the same host" (Rutledge et al. 1976). However, the work of McLaughlin and Vidrine (1987) showing that populations of *Psorophora columbiae* (Dyar and Knab) are reduced when host density is reduced demonstrates that the assumption of free-choice conditions in nature is not necessarily valid. Host availability is a limiting factor for populations of *Ps. columbiae*.

Curtis et al. (1987) found that results obtained with free-choice test methods were more variable than results obtained with comparable no-choice test methods. Subsequently, Klun and Debboun (2000) redesigned the American Society for Testing and Materials (1983) forearm test module to function as a no-choice test module.

In view of these considerations, we believe that in vitro test systems should be designed, or redesigned, to function in the no-choice mode. An additional advantage of the no-choice design is that the data obtained are analyzed by quantal methods of probit analysis, which are well known and widely used, whereas data obtained in free-choice tests are analyzed by graded response methods of probit analysis, which are little known and little used (Finney 1971).

Goldbeater's skin, derived from bovine large intestine, differs greatly from human skin, the substrate to which mosquito repellents are applied in practice. Actual skin is composed of the epidermis, which produces the stratum corneum and the skin pigments, and the dermis, a connective tissue containing blood vessels, lymph vessels, nerve endings, hair follicles, and the skin glands. It is reasonable that greater accuracy, precision, and reliability in in vitro test systems could be obtained by replacing goldbeater's skin with actual skin or a skinlike material (Rutledge et al. 1964). One approach would be to standardize a species of fresh

or frozen shaved mammal skin that is inexpensive and easily obtained. Alternatively, it might be possible to use a cultured human skin product such as TestSkin™ (Organogenesis, Cambridge, MA) or Skin2™ (Marrow-Tech, La Jolla, CA).

The operating temperature of the feeders in the in vitro bloodfeeding test system of Rutledge et al. (1976) is 37°C. (normal human body temperature), but the temperature of the skin, where mosquitoes interact with humans, is only 30–32°C. (Knols et al. 1994). It is reasonable that greater accuracy, precision, and reliability could be achieved in in vitro test systems by lowering the temperature of the feeders to a more natural level.

Finally, a major deficiency of the in vitro bloodfeeding test system is that it cannot be used to test the persistence of repellents on the skin because the blood in the feeders is stagnant and deteriorates rapidly to form a crust at the interface with the membrane. It is reasonable that longer tests could be accomplished if the feeders were redesigned as a flow-through or recirculating system that could be used in connection with a heart–lung machine, dialyzer (artificial kidney), or other suitable equipment.

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