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EVALUATION OF THE LABORATORY MOUSE MODEL FOR SCREENING TOPICAL MOSQUITO REPELLENTS[1]

L. C. RUTLEDGE,[2] R. K. GUPTA,[3] R. A. WIRTZ[4]
AND M. D. BUESCHER[5]

ABSTRACT. Eight commercial repellents were tested against *Aedes aegypti* 0 and 4 h after application in serial dilution to volunteers and laboratory mice. Results were analyzed by multiple regression of percentage of biting (probit scale) on dose (logarithmic scale) and time. Empirical correction terms for conversion of values obtained in tests on mice to values expected in tests on human volunteers were calculated from data obtained on 4 repellents and evaluated with data obtained on 4 others. Corrected values from tests on mice did not differ significantly from values obtained in tests on volunteers. Test materials used in the study were dimethyl phthalate, butopyronoxyl, butoxy polypropylene glycol, MGK Repellent 11®, deet, ethyl hexanediol, Citronyl®, and dibutyl phthalate.

INTRODUCTION

In 1987 a committee of the National Research Council recommended use of animal models in lieu of human subjects for screening potential mosquito repellents (National Research Council 1987). This recommendation was prompted by concern for the safety of human subjects who may be exposed to test materials for which toxicity information is inadequate or incomplete.

Several animal models have been used in testing mosquito repellents, but only 2 have been shown to predict accurately the results to be expected in tests on human subjects. Kasman et al. (1953) demonstrated that protection times obtained in tests on guinea pigs against *Aedes aegypti* (Linn.) were significantly correlated with the corresponding values obtained in tests on humans. Hill et al. (1979) demonstrated that minimum effective doses and protection times obtained in tests on hairless dogs against *Ae. aegypti* were significantly correlated with the corresponding values obtained in tests on humans.

Reifenrath and Rutledge (1983) published a dose–response method for testing repellents on laboratory mice against *Ae. aegypti.* Adaptations of the method were used in tests of repellents against *Xenopsylla cheopis* (Rothschild) (Siphonaptera: Pulicidae) (Mehr et al. 1984), *Diamanus montanus* (Baker) (Siphonaptera: Ceratophyllidae) (Rutledge et al. 1982), *Ornithodoros parkeri* Cooley (Acari: Argasidae) (Mehr et al. 1986), and *Dermacentor variabilis* (Say) (Acari: Ixodidae) (Rutledge, unpublished data).

The purpose of the present study was to evaluate the laboratory mouse model of Reifenrath and Rutledge (1983) and to calculate correction terms for conversion of results obtained in the test to corresponding values expected in tests on humans.

MATERIALS AND METHODS

Test insects: The University of California at San Francisco strain of *Ae. aegypti* was used in the study. The colony was maintained on white laboratory rabbits. Rearing conditions and procedures were as stated by Rutledge et al. (1978). Nulliparous females 5–15 days old were used in the tests.

Test materials: Repellents used in the study are shown in Table 1. All test materials were technical grade, commercially obtained.

Arm test: Repellents listed in Table 1 were tested on 7 individual human subjects. All subjects gave free and informed consent, and the investigators complied with all applicable laws and regulations on use of human subjects in research.

Tests were conducted in accordance with Standard E951-83 of the American Society for Testing and Materials (1983). Five 29-mm-diam circular test areas were outlined on the flexor surface of the forearm and treated at random with 0.025 ml of ethanol (control) and 4 serial dilutions of the test material in ethanol. A 4 × 5 × 18-cm plastic test cage containing 15 mosquitoes was applied to the forearm, and a slide was withdrawn to expose the test areas through matching holes in the floor of the cage. The number of mosquitoes biting in each test area at the end of 90 sec was recorded.

In initial trials, a range of low doses was se-

[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.

[2] 11 Circle Way, Mill Valley, CA 94941-3420.

[3] Headquarters, U.S. Army Research, Development, Acquisition and Logistics Command, Fort Detrick, Frederick, MD 21702-5012.

[4] Walter Reed Army Institute of Research, Washington, DC 20307-5100.

[5] 118 Yorkshire Drive, Williamsburg, VA 23185–3983.

Table 1. Test materials used in the study.

No.	Common/trade name	Chemical name
1	dimethyl phthalate	dimethyl phthalate
2	butopyronoxyl	butyl 3,4-dihydro-2,2-dimethyl-4-oxo-2*H*-pyran-6-carboxylate
3	dibutyl phthalate	di-*n*-butyl phthalate
4	butoxy polypropylene glycol	butoxypropanediol polymer
5	deet	*N,N*-diethyl-3-methylbenzamide
6	ethyl hexanediol	2-ethyl-1,3-hexanediol
7	Citronyl®	3-acetyl-2-(2-6-dimethyl-5-heptenyl)-oxazolidine
8	MGK Repellent 11®	1,5a,6,9,9a,9b-hexahydro-4a(4H)-dibenzofurancarboxaldehyde

lected for testing immediately after application, and a range of high doses was selected for testing 4 h after application. Tests were replicated 8–29 times (median = 14) at each range of doses. On completion of the tests, totals were obtained for the numbers of mosquitoes biting the treatments and the respective (0- or 4-h) controls. Treatment totals were converted to percentage of the respective control totals and subtracted from 100 to express the response in terms of the percentage of mosquitoes repelled. These values were converted to the corresponding probit values for analysis.

Test data were analyzed by multiple regression as described by Rutledge et al. (1985). The analysis employs the probit plane model of Busvine (1971) and Finney (1971):

$$Y = A + B_1X_1 + B_2X_2,$$

in which Y is the population response to the test material in probits, X_1 is the logarithm of the applied dose in mg/cm^2, X_2 is the elapsed time from time of application in hours, and A, B_1, and B_2 are the Y intercept and partial regression coefficients for dose and time, respectively.

Mouse test: Repellents listed in Table 1 were tested on generic 7–10-day-old white laboratory mice. In conducting the research described, the investigators adhered to National Institutes of Health Publication 85-23, *Guide for the care and use of laboratory animals.*

Tests were conducted as described by Reifenrath and Rutledge (1983). Five mice were treated at random with ethanol (control) and 4 serial dilutions of the test material in ethanol. Treatments were applied with a pipet over the whole body to point of runoff. Treated mice were transferred to a 30 × 30 × 30-cm test cage containing 100 mosquitoes. The test cage was a standard aluminum frame mosquito cage (American Biological Supply Company, Baltimore, MD) modified by replacing the aluminum floor with additional screening. During the test, the cage was supported 10 cm above the surface of the work table to permit circulation of air through all parts of the cage. The number of mosquitoes biting each mouse was recorded at 2-min intervals for a 30-min period. Totals for each mouse were obtained at the end of the test.

In initial trials, a range of low doses was selected for testing immediately after application, and a range of high doses was selected for testing 4 h after application. Tests were replicated 2–9 times (median = 3) at each range of doses. On completion of the tests, totals were obtained for the numbers of mosquitoes biting the treatments and the respective (0- or 4-h) controls. Treatment totals were converted to percentage of the respective control totals and subtracted from 100 to express the response in terms of the percentage of mosquitoes repelled. These values were converted to the corresponding probit values for analysis.

Test data were analyzed by multiple regression as described for the arm test. In this report, lowercase letters will be used to distinguish mouse test data from arm test data:

$$y = a + b_1x_1 + b_2x_2.$$

Derivation of correction terms: In this study, different units of dose were employed in the arm test (mg of repellent per cm^2 of skin) and the mouse test (percentage concentration of solution applied). Accordingly, a dose conversion term was determined in the preliminary analysis (Appendix). The purpose of the dose conversion term was to permit comparison of arm test data with mouse test data on a mg/cm^2 basis.

Empirical correction terms then were calculated using dose-corrected mouse test data obtained in tests of repellents 1–4 only. The purpose of the empirical correction terms was to permit conversion of mouse test data to arm test data.

Preliminary analysis indicated that an additive model was more efficient than a multiplicative model or a mixed model in terms of predictive efficacy. Accordingly, empirical corrections to a, b_1, and b_2, denoted C_a, C_{b1}, and C_{b2}, were cal-

Table 2. Multiple regression data for 4 repellents tested against *Aedes aegypti* on the forearm of humans and on laboratory mice.

	Tests on forearm			Tests on mice[2]					
No.[1]	A	B_1	B_2	a	b_1	b_2	A − a	$B_1 - b_1$	$B_2 - b_2$
1	10.7395	2.3575	−1.1286	7.1245	2.2700	−0.2597	3.6150	0.0875	−0.8689
2	11.6053	2.7679	−1.0612	8.3699	1.7333	−0.3562	3.2354	1.0346	−0.7050
3	11.8354	2.9168	−1.7403	6.5670	1.9233	−0.4913	5.2684	0.9935	−1.2490
4	6.0978	2.1483	−0.4943	5.3660	1.4873	−0.1840	0.7318	0.6610	−0.3103
Mean							3.2126	0.6942	−0.7833
Standard error							0.9373	0.2188	0.1945

[1] See Table 1 for identification of repellents.

[2] Values of a were adjusted to permit comparison with A on the same (mg/cm²) dose basis (Appendix). To obtain corresponding values on the original (ml/100 ml) dose basis, subtract $0.1540(b_1)$ from the figures shown. Values of b_1 and b_2 are not affected by the dose conversion.

culated to provide expected values of A, B_1, and B_2 such that $A' = a + C_a$, $B_1' = b_1 + C_{b1}$, and $B_2' = b_2 + C_{b2}$. C_a, C_{b1}, and C_{b2} were estimated as the means of A − a, $B_1 - b_1$, and $B_2 - b_2$ for repellents 1–4.

Verification: Correction terms derived in the study were evaluated with test data obtained on repellents 5–8.

Rounding error: Data herein are reported to 4 decimal places to permit cross-checking by the reader without excessive rounding error due to application of the logarithmic and probit transformations. This convention is not intended to imply that the data are accurate to 4 significant figures.

RESULTS AND DISCUSSION

Arm tests: Results from the arm tests are shown in columns 2–4 of Table 2 for repellents 1–4 and in columns 2–4 of Table 3 for repellents 5–8. Values shown are estimates of the Y intercept (A) and the partial regression coefficients for dose (B_1) and time (B_2) obtained in the respective multiple regression analyses. All multiple correlation coefficients were significant at the 5% level except that for repellent 4 (butoxy polypropylene glycol).

Values of A (column 2 of Tables 2 and 3) represent the expected response to the repellent, in probits, when X_1 and X_2 are 0, i.e., the expected response when a unit dose of 1 mg/cm² (log 1 = 0) is tested immediately (0 h) after application. Values of B_1 (column 3 of Tables 2 and 3) are positive, reflecting the expected increase in the proportion of mosquitoes repelled with increasing dose. Values of B_2 (column 4 of Tables 2 and 3) are negative, reflecting the expected decrease in the proportion of mosquitoes repelled with increasing time from the time of application.

Mouse tests: Results from the mouse tests are shown in columns 5–7 of Table 2 for repellents 1–4 and in columns 5–7 of Table 3 for repellents 5–8. Values shown are estimates of the y intercept (a) and partial regression coefficients for dose (b_1) and time (b_2) obtained in the respective multiple regression analyses. All multiple correlation coefficients were significant at the 5% level. Interpretation of a, b_1, and b_2 is analogous to that given for A, B_1, and B_2.

Table 3. Observed and corrected multiple regression data for 4 additional repellents tested against *Aedes aegypti* on the forearm of humans and on laboratory mice.

				Tests on mice[2]					
	Tests on forearm			Observed			Corrected		
No.[1]	A	B_1	B_2	a	b_1	b_2	A′	B_1'	B_2'
5	13.1240	3.3807	−1.6084	10.1253	2.3350	−0.5521	13.3379	3.0292	−1.3354
6	12.3355	3.2113	−1.6833	9.1543	2.0506	−0.6927	12.3669	2.7448	−1.4760
7	8.1515	1.3642	−0.5092	6.7991	1.1483	−0.0505	10.0117	1.8425	−0.8338
8	12.5241	3.1963	−1.2919	7.8345	2.6367	−0.4752	11.0471	3.3309	−1.2585

[1] See Table 1 for identification of repellents.

[2] Values of a were adjusted to permit comparison with A on the same (mg/cm²) dose basis (Appendix). To obtain corresponding values on the original (ml/100 ml) dose basis, subtract $0.1540(b_1)$ from the figures shown. Values of b_1 and b_2 are not affected by the dose conversion.

Table 4. Comparison of corrected and uncorrected values of multiple regression parameters obtained in tests on laboratory mice with values obtained in tests on humans.

Before correction				After correction			
Comparison	t	df	P	Comparison	t	df	P
A vs. a	4.477	3	<0.05	A vs. A′	−0.230	3	>0.05
B_1 vs. b_1	3.398	3	<0.05	B_1 vs. B_1'	0.234	3	>0.05
B_2 vs. b_2	−6.204	3	<0.05	B_2 vs. B_2'	−0.353	3	>0.05

Correction terms: Column 8 of Table 2 shows the observed differences between the Y and y intercepts in the arm test and the mouse test (A − a) for repellents 1–4. Values ranged from 0.7318 for repellent 4 (butoxy polypropylene glycol) to 5.2684 for repellent 3 (dibutyl phthalate), with mean of 3.2126 and standard error of 0.9373. The largest observed deviation from the mean, −2.4808 for repellent 4 (butoxy polypropylene glycol), was not statistically significant ($z = -1.32$; $P > 0.05$). On this basis, the observed differences between the arm test and mouse test intercepts (A − a) were regarded as a sample from a normal distribution, and the mean of the sample (3.2126) was regarded as an unbiased estimate of the correction term C_a.

It was noted that the mean of the arm test intercept (10.0695) was significantly greater than the mean of the mouse test intercept (6.8568) ($t = 3.427$, df = 3, $P < 0.05$). This result indicates that the test materials were more effective against *Ae. aegypti* in the arm test than they were in the mouse test. Differences in the test systems (host species, size of the test population, size of the test cage, etc.) may collectively account for the observed difference in effectiveness.

Column 9 of Table 2 shows the observed differences between the coefficients of regression for dose in the arm test and the mouse test ($B_1 - b_1$) for repellents 1–4. Values ranged from 0.0875 for repellent 1 (dimethyl phthalate) to 1.0346 for repellent 2 (butopyronoxyl), with mean of 0.6942 and standard error of 0.2188. The largest observed deviation from the mean, −0.6067 for repellent 1 (dimethyl phthalate), was not statistically significant ($z = -1.39$; $P > 0.05$). On this basis, the observed differences between the arm test and mouse test coefficients ($B_1 - b_1$) were regarded as a sample from a normal distribution, and the mean of the sample (0.6942) was regarded as an unbiased estimate of the correction term C_{b1}.

It was noted that the mean of the arm test coefficient (2.5476) was significantly greater than the mean of the mouse test coefficient (1.8535) $t = 3.172$, df = 3, $P = 0.051$). Because the reciprocal of the slope of the dose–response line is equal to the standard deviation of the response to the test material, this result indicates that responses of *Ae. aegypti* were less variable in the arm test than in the mouse test. Differences in the test systems (host species, size of the test population, size of the test cage, etc.) may collectively account for the observed difference in the variation of the response in the 2 tests.

Column 10 of Table 2 shows the observed differences between coefficients of regression for time in the arm test and the mouse test ($B_2 - b_2$) for repellents 1–4. Values ranged from −1.2490 for repellent 3 (dibutyl phthalate) to −0.3103 for repellent 4 (butoxy polypropylene glycol), with mean of −0.7833 and standard error of 0.1945. The largest observed deviation from the mean, 0.4730 for repellent 4 (butoxy polypropylene glycol), was not statistically significant ($z = 1.44$; $P > 0.05$). On this basis, the observed differences between the arm test and the mouse test coefficients ($B_2 - b_2$) were regarded as a sample from a normal distribution, and the mean of the sample (−0.7833) was regarded as an unbiased estimate of the correction term C_{b2}.

It was noted that the mean of the arm test coefficient (−2.5476) was significantly smaller than the mean of the mouse test coefficient (−0.3228) ($t = -4.027$, df = 3, $P < 0.05$). This result indicates that the test materials were less persistent in the arm test than in the mouse test. Differences in host species (size, body temperature, skin thickness, perspiration, etc.) may collectively account for the observed difference in persistence.

Verification: Table 3 shows observed values of A, B_1, and B_2 from tests of repellents 5–8 on the forearm (columns 2–4) and observed values of a, b_1, and b_2 from tests of the same repellents on mice (columns 5–7). Expected values of A, B_1, and B_2 are shown in columns 8–10. Expected values were obtained by adding the correction terms $C_a = 3.2126$, $C_{b1} = 0.6942$, and $C_{b2} = -0.7833$ to the corresponding values of a, b_1, and b_2. Variances (V) of expected values can be obtained as $V(A') = V(a) + V(C_a)$, $V(B_1') = V(b_1) + V(C_{b1})$, and $V(B_2) = V(b_2) + V(C_{b2})$ if needed in future studies.

Expected values of A, B_1, and B_2 for repellents 5–8 were tested by Student's t (Table 4). Al-

though values of a, b_1, and b_2 obtained in the mouse test differed significantly from values of A, B_1, B_2 obtained in the arm test, expected values obtained by applying correction terms to a, b_1, and b_2 did not. On this basis, it was concluded that the correction terms C_a, C_{b1}, and C_{b2} were effective in converting values obtained in tests of repellents 5–8 on laboratory mice to the corresponding values expected in tests on humans.

Estimates of the percentage of mosquitoes repelled 0 and 4 h after application of 0.1 and 1 mg/cm² of repellents 5–8 are shown in Table 5. Estimates provided by corrected mouse test data were most accurate in the low (≤5%) and high (≥95%) ranges. This reflects the statistical distribution of the probit values employed to transform the sigmoid dose–response curve to a straight line. Interest has traditionally centered on the high (≥95%) range for practical reasons.

Values of the dose conversion term (Appendix) and the correction terms C_a, C_{b1}, and C_{b2} were refined for future use by combining the data obtained on repellents 1–4 and 5–8. The revised value of the dose conversion term was −0.1411 with standard error of 0.0245. Accordingly, a dose of 1 ml/100 ml (1%) in the mouse test is equivalent to a dose of 0.7226 mg/cm² in the arm test (antilog −0.1411 = 0.7226), and a dose of 1 mg/cm² in the arm test is equivalent to a dose of 1.3839 ml/100 ml (1.3839%) in the mouse test (1/0.7226 = 1.3839). Revised values of the corrections to a, b_1, and b_2 were C_a = 3.1592, C_{b1} = 0.7198, and C_{b2} = −0.8069 with standard errors of 0.5391, 0.1438, and 0.1097, respectively.

Because the skin is the site of action of topical repellents, it should be noted that there are significant differences in the skins of mice and humans with regard to pigmentation, hair, and presence or absence of sweat glands (Sokolov 1982). These and other differences in skin structure and function undoubtedly contribute to the differences in effectiveness and persistence reflected by the correction terms derived in the study.

In toxicology, methods have been developed for extrapolating data obtained in tests of chemicals on animals to problems of carcinogenesis, mutagenesis, and teratogenesis in humans (Hogan and Hoel 1989). Many of the concepts employed, including the concepts of threshold dose and the dose–response relationship, are identical to those employed in modern repellent screening programs. From this viewpoint, the present study may represent a beginning for further development along the lines pioneered in toxicology.

Methods utilizing laboratory mice in large-scale, semi-automated screening programs might eventually be developed, using concepts developed in the present study to relate the results obtained to those expected in tests on human subjects. Kashin and Kardatzke (1969) and Kashin and Arneson (1969) described a method using an electronic apparatus to record the numbers of bites and time of biting on repellent-treated mice. Alternatively, the method of Lal et al. (1963), using fluorescent dyes administered intravenously to mark mosquitoes biting treated and untreated mice, might be updated with systems to automatically count the marked mosquitoes to provide an efficient, rapid screening method.

Table 5. Comparison of estimates of percentage of repellency obtained in tests on mice with estimate obtained in tests on arms of humans.

Repellent[1]	Arm test	Mouse test	Difference
0.1 mg/cm², 0 h after application			
5	100.0	100.0	0.0
6	100.0	100.0	0.0
7	96.3	99.9	−3.6
8	100.0	99.7	0.3
Mean[2]			2.0
0.1 mg/cm², 4 h after application			
5	4.5	48.7	−44.2
6	0.5	10.0	−9.5
7	40.2	43.4	−3.2
8	20.1	1.0	19.1
Mean[2]			19.0
1 mg/cm², 0 h after application			
5	100.0	100.0	0.0
6	100.0	100.0	0.0
7	99.9	100.0	−0.1
8	100.0	100.0	0.0
Mean[2]			0.0
1 mg/cm², 4 h after application			
5	95.5	99.9	−4.4
6	72.6	92.8	−20.2
7	86.7	95.3	−8.6
8	99.1	84.4	14.7
Mean[2]			12.0

[1] See Table 1 for identification of repellents.
[2] Mean of absolute values.

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APPENDIX

The purpose of this appendix is to provide additional information on the experimental and analytical methods employed in the study. The information provided is not required for a general understanding of the report.

The correction term C_a was estimated by the difference of intercepts ($A - a$), in which units of the minuend and subtrahend (probits) were the same. Accordingly, the unit of C_a is the probit, and application of C_a in the equation $A' = a + C_a$ leaves the unit unchanged, as required in estimating A from a.

The correction term C_{b1} was estimated by the difference of the partial regression coefficients for dose ($B_1 - b_1$), which were in units of probits/unit dose. The unit of dose employed in the arm test was mg of repellent per cm^2 of skin (mg/cm^2), and the unit of dose employed in the mouse test was the percentage concentration of solution applied (ml/100 ml). Accordingly, C_{b1} includes components for both the interspecies conversion and the unit conversion. In particular, it is clear from the empirical equation $B_1' = b_1 + C_{b1}$ that C_{b1} includes an additive component for conversion of log ml/100 ml to log mg/cm^2, as required in estimating B_1 from b_1.

It can be shown as follows that the units in question are inherently equivalent: In the arm test a sufficient weight of repellent is applied in 0.025 ml of solution to provide a dose of $\log^{-1} X_1$ mg/cm^2 over an area of 6.6 cm^2 of skin. If w is the weight of repellent applied in mg and d is the density of the repellent in mg/ml, then the volume of repellent applied is w/d ml, and the dose can be expressed as (w/d ml)/(0.025 ml), or (0.4[w/d] ml)/(100 ml), which is in the unit of dose employed in the mouse test (ml of repellent per 100 ml of solution).

Conversely, in the mouse test a concentration of repellent equal to $\log^{-1} x_1$ ml of repellent per 100 ml of solution is applied to the skin of a 7–10-day-old mouse to point of runoff. If s is the average surface area of a 7–10-day-old mouse in cm^2 and V is the volume of solution in ml that can be applied to point of runoff over an area of s cm^2, then the solution is applied to the mouse at a rate of (V ml)/(s cm^2). The volume, V, of solution applied includes a lesser volume, v, of repellent that is equal to $([\log^{-1} x_1]/100)V$ ml and is applied at the rate of (v ml)/(s cm^2). If d is the density of the repellent in mg/ml, then the weight of repellent applied is dv mg, and the rate of application is (dv mg)/(s cm^2), or dv/s mg/cm^2, which is in the unit of dose employed in the arm test (mg/cm^2).

It can be shown algebraically that the increment of dose required to increase the population

response to the test material by one unit (one probit) is numerically equal to $1/B_1$ in the arm test and to $1/b_1$ in the mouse test. Because these increments produce equal responses in the test population, they may be regarded as equal in terms of rate of application, and the difference $1/B_1 - 1/b_1$ may be attributed to the difference in the units of measurement employed in the arm test and the mouse test. The quantity $1/B_1 - 1/b_1$ is the amount by which the unit dose in the mouse test must be increased to obtain a response equal to that provided by the unit dose in the arm test.

The effect of adding the quantity $1/B_1 - 1/b_1$ to x_1 in the multiple regression analysis is to reduce the value of the y intercept, a, by an amount equal to $b_1(1/B_1 - 1/b_1)$. Values of b_1 and b_2 are not affected. Accordingly, a dose conversion term, k, can be defined for mouse test data such that $k = b_1(1/B_1 - 1/b_1)$, the amount to be subtracted from the value of a in the dose units of x_1 to obtain the value of a in the dose units of X_1. The variance of a is not affected by the conversion.

The value of the quantity $1/B_1 - 1/b_1$ was estimated as the mean of the separate estimates available in the data obtained on repellents 1–4 (Table 2). The value so obtained was −0.1540 with standard error of 0.0466. Because this value is logarithmic, physical interpretation is in terms of the antilog, which is 0.7015 with 95% confidence limits of 0.5532 and 0.8498: A dose of 1 ml/100 ml (1%) in the mouse test is equivalent to a dose of 0.7015 mg/cm^2 in the arm test, and, conversely, a dose of 1 mg/cm^2 in the arm test is equivalent to a dose of 1.4255 ml/100 ml (1.4255%) in the mouse test (1/0.7015 = 1.4255).

The correction term C_{b2} was estimated by the difference of partial regression coefficients for time ($B_2 - b_2$), in which units of the minuend and subtrahend (probits/h) were the same. Accordingly, the unit of C_{b2} is probits/h, and application of C_{b2} in the equation $B_2' = b_2 + C_{b2}$ leaves the unit unchanged, as required in estimating B_2 from b_2.