Annex IV

Analyses to Determine Representative EC3 Values

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1.0 Introduction

The analyses of the 63 murine local lymph node (LLNA) and human sensitizers detailed in **Section 6.0** of this background review document (BRD) include both linear regressions and Spearman correlations of the log-transformed LLNA EC3 values (i.e., estimated concentration of a substance expected to produce a stimulation index of 3, the threshold value for a substance to be considered a sensitizer in the LLNA) and human DSA_{05} values (induction dose per skin area, in $\mu g/cm^2$, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population), both in units of $\mu g/cm^2$. This annex describes the analyses performed to evaluate various approaches to calculate the geometric mean EC3 values for substances with multiple LLNA results. The approaches explored (1) the use of negative LLNA results for substances that also produced positive results (i.e., how to account for discordant negative results), (2) the use of vehicle-specific LLNA results for substances that had tests in multiple vehicles, and (3) the use of LLNA results from nonstandard protocols (**Section 5.1** of the BRD). Geometric mean DSA_{05} values were calculated using all available DSA_{05} values for each substance with multiple values.

2.0 Methods and Results

2.1 Combining LLNA Results Tested in Different Vehicles

Although two important factors that contribute to skin sensitization (i.e., the ability of the test substance to traverse the stratum corneum and reach the viable epidermis and the efficiency of Langerhans cell migration) are susceptible to vehicle effects (Basketter et al. 2001; Lea et al. 1999; McGarry 2007; Wright et al. 2001), others have noted that vehicle may have little impact on the accuracy of hazard identification in properly conducted standard test methods (Kimber et al. 2003). With respect to the LLNA potency analyses, while vehicle may be an important determinant of the EC3 value, it may not be important for every substance tested and therefore may have no overall effect on the linear regression analyses that include over 60 substances. To determine if multiple results for individual substances should be evaluated by vehicle (averaging EC3 values for each vehicle and then averaging the vehicle means) or without consideration of vehicle (averaging all EC3 values regardless of vehicle), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) first evaluated whether there was a significant vehicle effect on LLNA EC3 values for the substances in the current database. **Table C-IV-1** shows the 27 vehicles represented in the NICEATM LLNA potency database and the number of LLNA tests for each vehicle. The LLNA vehicle was not specified for 29 tests.

The first analysis was a two-way analysis of variance (ANOVA) (Steel et al. 1997) with substance and vehicle as the factors that influence the EC3 value. EC3 data from four major vehicles represented in the database were used in the analysis: (1) acetone, (2) acetone: olive oil (4:1) (AOO), (3) dimethyl sulfoxide (DMSO), and (4) dimethyl formamide (DMF). All of these vehicles were mentioned as commonly used vehicles in the 1998 Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluation of the LLNA (ICCVAM 1999). AOO, DMSO, and DMF are recommended vehicles in Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 (OECD 2002), the U.S. Environmental Protection Agency (EPA) Health Effects TG 870.2600 (EPA 2003), and the updated ICCVAM-recommended LLNA protocol (ICCVAM 2009). For the two-way ANOVA, all negative LLNA tests that used concentrations that were less than positive tests for the same substance in the same vehicle (n = 10) were deleted. All other negative LLNA tests (i.e., negative at maximum doses that produced positive results in other LLNA tests) were assigned an EC3 = 110% as an arbitrary value for the EC3 (which exceeds the maximum possible value of 100% for a positive response) in order to maximize the available database. Data for substances that were tested in a single vehicle were deleted. The analysis

used data for 28 substances with 261 EC3 values. The two-way ANOVA on the log-transformed data indicated that vehicle was not a significant factor in determining the EC3 value ($F = 1.4758$, $p = 0.2400$) for tests with the four vehicles used in the analysis.

Table C-IV-1 Number of Tests for Each LLNA Vehicle

LLNA Vehicle	Number of Tests	% Total Tests
Acetone: olive oil (4:1)	319	47.9%
Dimethyl formamide	72	10.8%
Pluronic L92	50	7.5%
Ethanol/diethyl phthalate (1:3)	50	7.5%
Dimethyl sulfoxide	45	6.7%
Acetone	30	4.5%
Not specified[1]	29	4.4%
Ethanol/diethyl phthalate (3:1)	9	1.4%
Methyl ethyl ketone	9	1.4%
Propylene glycol	7	1.0%
Diethyl phthalate	6	0.9%
Ethanol	5	0.8%
Ethanol/diethyl phthalate (3:1) + 0.1% Trolox C	4	0.6%
Ethanol/diethyl phthalate (3:1) + acetone: olive oil (3:1) mix[2]	4	0.6%
Water	4	0.6%
Hydroxypropyl cellulose in methanol	4	0.6%
Ethanol/diethyl phthalate (3:1) + 0.1% tocopherol	4	0.6%
Ethanol (10%)	2	0.3%
Ethanol (50%)	2	0.3%
Ethanol/diethyl phthalate (3:1) + 2% tocopherol	2	0.3%
Petrolatum	2	0.3%
Acetone/Water (3:1)	1	0.2%
Dimethyl formamide/water	1	0.2%
Dimethyl sulfoxide/water (9:1)	1	0.2%
Ethanol (25%)	1	0.2%
Ethanol (30%)	1	0.2%
Ethanol (80%)	1	0.2%
Methyl ethyl ketone: olive oil (4:1)	1	0.2%
Grand Total	**666**	

Abbreviations: LLNA = murine local lymph node assay.

[1] Information on the vehicle used was not provided.

[2] Mix = 0.3% butylated hydroxytoluene/tocopherol/eugenol.

Another two-way ANOVA was then performed using results from all vehicles with five or more tests. Again, substances that were tested in a single vehicle were deleted, as were the negative tests that used concentrations that were less than positive tests for the same substance and vehicle (n = 10). As above, the remaining negative LLNA tests were assigned an EC3 = 110%. The analysis included data for 41 substances, 11 vehicles, and 376 EC3 values. The two-way ANOVA of the log-transformed data indicated that vehicle was a significant factor in determining the EC3 value ($F = 4.0801$, $p = 0.0002$) for vehicles with at least five LLNA tests.

To determine which vehicles were responsible for the significant vehicle effect on the EC3 value, a number of additional two-way ANOVAs were performed for variations of the dataset that excluded one or more vehicles. Excluding propylene glycol and Pluronic L92 removed the significant effect of vehicle ($F = 1.75377$, $p = 0.1000$). The analysis used data for 41 substances, nine vehicles, and 352 EC3 values. Propylene glycol and Pluronic L92 tests accounted for only a small part of the 376-test dataset: 4.5% (17/376) and 1.9% (7/376), respectively.

By excluding only two vehicles, which accounted for a small proportion of the data, there was no significant vehicle effect on EC3 values in the current database. Thus, an EC3 versus DSA_{05} linear regression analysis was performed on the geometric mean EC3 value for each substance regardless of vehicle (see **Section 2.4.1**). A second linear regression was performed by using a geometric mean EC3 value for each substance that was calculated from the geometric mean EC3 values for each vehicle-substance combination (i.e., a geometric mean of the vehicle-substance geometric mean EC3 values). These two linear regressions were compared to provide additional evidence that vehicle has no effect on EC3 values in the current database. Finally, the optimal regression was repeated without propylene glycol and Pluronic L92 to confirm that there is no statistically significant vehicle effect on the EC3 versus DSA_{05} regression (see **Section 2.4.2**).

2.2 Combining LLNA Results for Substances With Both Sensitizer and Nonsensitizer Data

Some substances with multiple LLNA results have both sensitizer (positive) and nonsensitizer (negative) outcomes among the test results. In determining a representative EC3 value for such a substance, how should negative LLNA results be used? Negative LLNA test results could be replaced by an EC3 value that is unattainable in practice and averaged in with the positive tests. Negative LLNA test results could also simply be ignored. Then the EC3 values for only the positive tests for a given substance would be averaged. EC3 versus DSA_{05} linear regressions using geometric means were performed using two approaches for calculating representative EC3 values for each substance: (1) ignoring negative results, and (2) replacing negative results with 110% (see **Section 2.4**).

2.3 The Effect of Nonstandard Protocols on LLNA Results

To address the question of whether LLNA results from nonstandard protocols were different from LLNA results using the standard LLNA protocol (Dean et al. 2001; ICCVAM 1999; OECD 2002), a two-way ANOVA was performed using substance and protocol as the variable factors for the EC3 value. The database for this analysis included 656 LLNA results (10 negative results that used concentrations lower than those required for positive results for the same substance in other tests were excluded). The remaining negative LLNA results were replaced with EC3 values of 110% so that they could be used in the analysis. The analysis included 196 substances and considered three protocol groups: standard (73% [479/656]), nonstandard (18% [120/656]), and not reported (9% [57/656]). The two-way ANOVA of the log-transformed results showed that protocol had no effect on EC3 values ($F = 1.3790$, $p = 0.2600$). To determine whether protocol affects the EC3 versus DSA_{05} linear regression, the optimum regression was repeated using only EC3 results from the standard protocol (see **Section 2.4.2**).

2.4 Correlation of EC3 with DSA_{05}

The analyses to establish the relationship of EC3 and DSA_{05} values included linear regressions on the log-transformed data expressed in units of $\mu g/cm^2$ and Spearman correlations. Note that the regressions and correlations use only substances that produced positive responses in the LLNA and in human maximization tests (HMT) and/or human repeat-insult patch tests (HRIPT). Although there were 65 substances that produced positive LLNA responses and positive HMT/HRIPT responses, nickel salts and streptomycin were excluded from the regressions because the most prevalent LLNA responses were negative (8/10 tests for nickel salts and 4/5 tests for streptomycin). Thus, the data available for the linear regressions and correlations include the 63 substances that yielded positive results in both the LLNA and human tests.

2.4.1 Approaches for Combining Multiple LLNA Results

The two major approaches for combining multiple results for EC3 and DSA_{05} values for individual substances were to use: (1) the most potent values (i.e., lowest EC3 and DSA_{05}) or (2) the geometric mean values. Other modifications to the geometric mean regression include two approaches to deal with negative LLNA results for substances that also produced positive results (i.e., how to use discordant negative results): (1) ignore the discordant negative results or (2) replace the discordant negative LLNA results with 110% (i.e., 27500 $\mu g/cm^2$). (Note: neither method for discordant negative results is applicable to the regression that uses the most potent values for each substance.) Additional modifications to the geometric mean regression include two approaches to using the vehicle-specific LLNA results for substances that had tests in multiple vehicles: (1) ignore the vehicle when calculating the geometric mean (i.e., pool all of the EC3 values) or (2) calculate a geometric mean of the results for each vehicle (for each substance) and then calculate the geometric mean of the vehicle-specific means for each substance. (Note: this computation is not applicable to the linear regressions using the most potent values for each substance.) For comparison, the linear regression using the optimal approach was repeated to confirm the lack of vehicle effect on the EC3 value by excluding the two vehicles that produced the significant vehicle effect in the two-way ANOVA, propylene glycol and Pluronic L92 (see **Section 2.4.2**). The linear regression using the optimal approach was also repeated to confirm the lack of a protocol effect by using only EC3 results from the standard LLNA protocol (Dean et al. 2001; ICCVAM 1999; OECD 2002) (see **Section 2.4.2**).

The linear regression results are shown in **Table C-IV-2**. Regression 1, which used the most potent results for EC3 and DSA_{05}, produced a slightly lower R^2 (0.382 versus 0.448) than regression 2, which used geometric mean EC3 and DSA_{05} values for substances with multiple results (see **Figure C-IV-1**). For regression 2, the geometric means were calculated across vehicle (i.e., potential vehicle effects were ignored) and discordant negative results were not included in the calculation (i.e., negatives were ignored). The geometric mean regression (3) that combined EC3 values regardless of vehicle and replaced discordant negative LLNA results with values of 27500 $\mu g/cm^2$ (i.e., equivalent to EC3 = 110%) was similar to the geometric mean regression (2) that did not use negative LLNA test results in the geometric mean EC3 values. This similarity is because only 10 negative LLNA results for six substances were replaced with 27500 $\mu g/cm^2$ in regression 3. Regressions 4 and 5 are similar to regressions 2 and 3, respectively, but use a different approach to combining multiple EC3 values. For regressions 4 and 5, a geometric mean was calculated for the EC3 values for each vehicle-substance combination, and then a geometric mean of those combination means was calculated for each substance. Regression 4 had a slightly lower slope than regression 2 (0.718 versus 0.742), possibly because the 10 discordant LLNA negative results exerted more influence on the regression when vehicle-specific results were averaged. In any case, the standard errors for the slopes and y-intercepts easily overlap for all of the geometric mean regressions (i.e., regressions 2, 3, 4, and 5). **Figure C-IV-2** graphically shows the similarity of these regressions. Thus, the inclusion (by

Table C-IV-2 Linear Regression and Correlation Results[1]

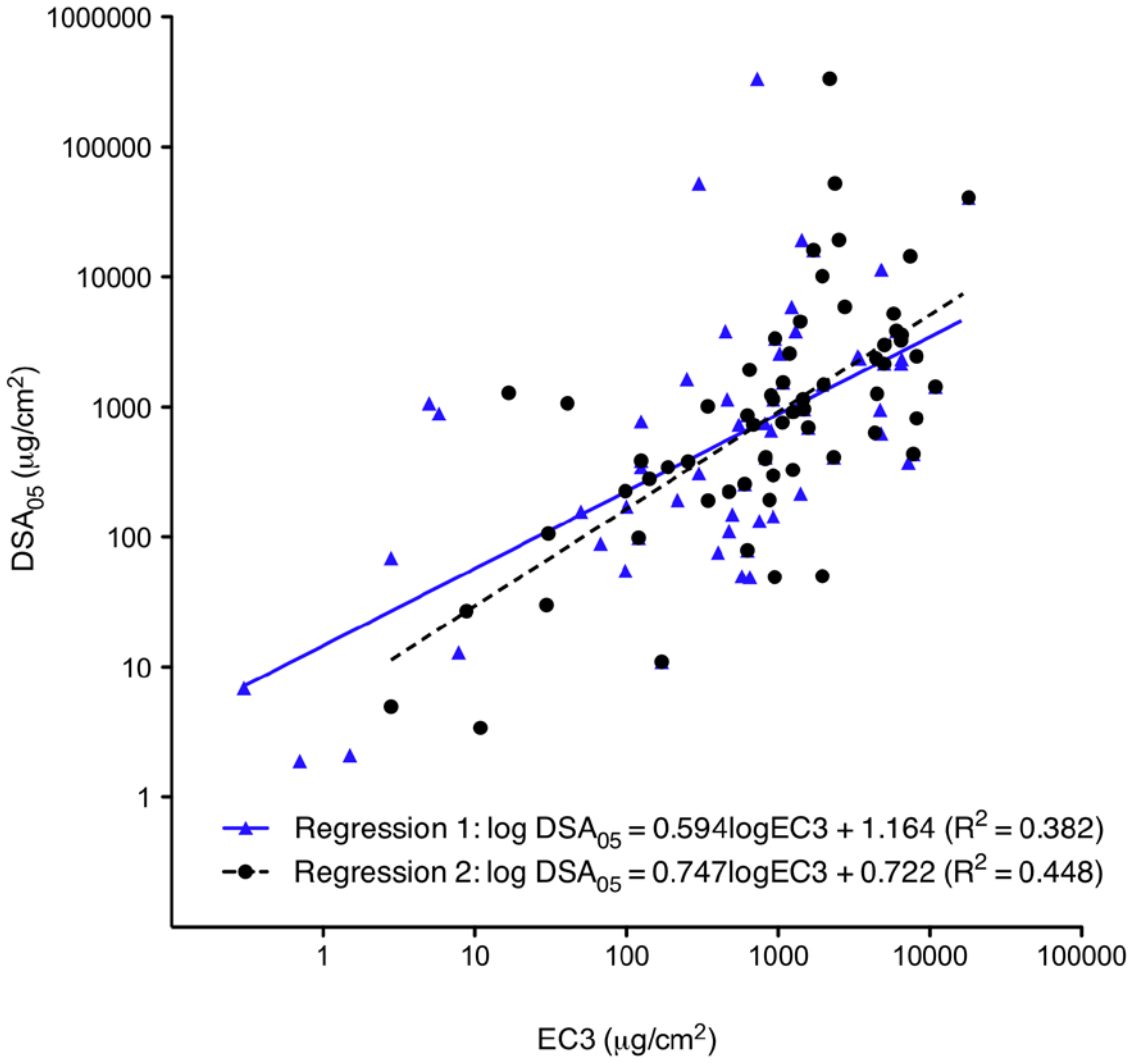
Description of Regression/Correlation	N	Linear Regression				Spearman Correlation	
		Slope (µg/cm^2)	Y-intercept	R^2	P-value	r	P-value
Standard, Nonstandard, and Unreported Protocols							
1) Most potent EC3 versus most potent DSA_{05}	63	0.594 ± 0.097	1.164 ± 0.275	0.382	<0.0001	0.594 (0.400-0.737)	<0.0001
2) Geometric mean EC3 versus geometric mean DSA_{05} – (vehicles ignored, negatives ignored)	**63**	**0.747 ± 0.106**	**0.722 ± 0.322**	**0.448**	**<0.0001**	**0.692 (0.530-0.804)**	**<0.0001**
3) Geometric mean EC3 (vehicles ignored, negatives = 27500 µg/cm^2) versus geometric mean DSA_{05}	63	0.742 ± 0.105	0.712 ± 0.322	0.451	<0.0001	0.692 (0.531-0.805)	<0.0001
4) Geometric mean EC3 (geometric mean vehicles, negatives = 27500 µg/cm^2) versus geometric mean DSA_{05}	63	0.718 ± 0.110	0.765 ± 0.338	0.414	<0.0001	0.646 (0.468-0.773)	<0.0001
5) Geometric mean EC3 (geometric mean vehicles, negatives ignored) versus geometric mean DSA_{05}	63	0.774 ± 0.107	0.639 ± 0.324	0.463	<0.0001	0.678 (0.512-0.796)	<0.0001
6) Optimal regression/correlation (3) repeated without propylene glycol and Pluronic L92 results	63	0.732 ± 0.104	0.773 ± 0.316	0.446	<0.0001	0.692 (0.531-0.805)	<0.0001
Standard Protocols							
7) Optimal regression/correlation (3) repeated with only the standard protocol results	54	0.701 ± 0.121	0.857 ± 0.353	0.393	<0.0001	0.642 (0.4495-0.780)	<0.0001

Boldface text highlights the optimal geometric mean linear regression.

Abbreviations: DSA_{05} = induction dose per skin area, in µg/cm^2, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, which is the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay; N = number of substances.

[1] Linear regressions and Spearman correlations used only the substances that were positive in both the LLNA and human tests.

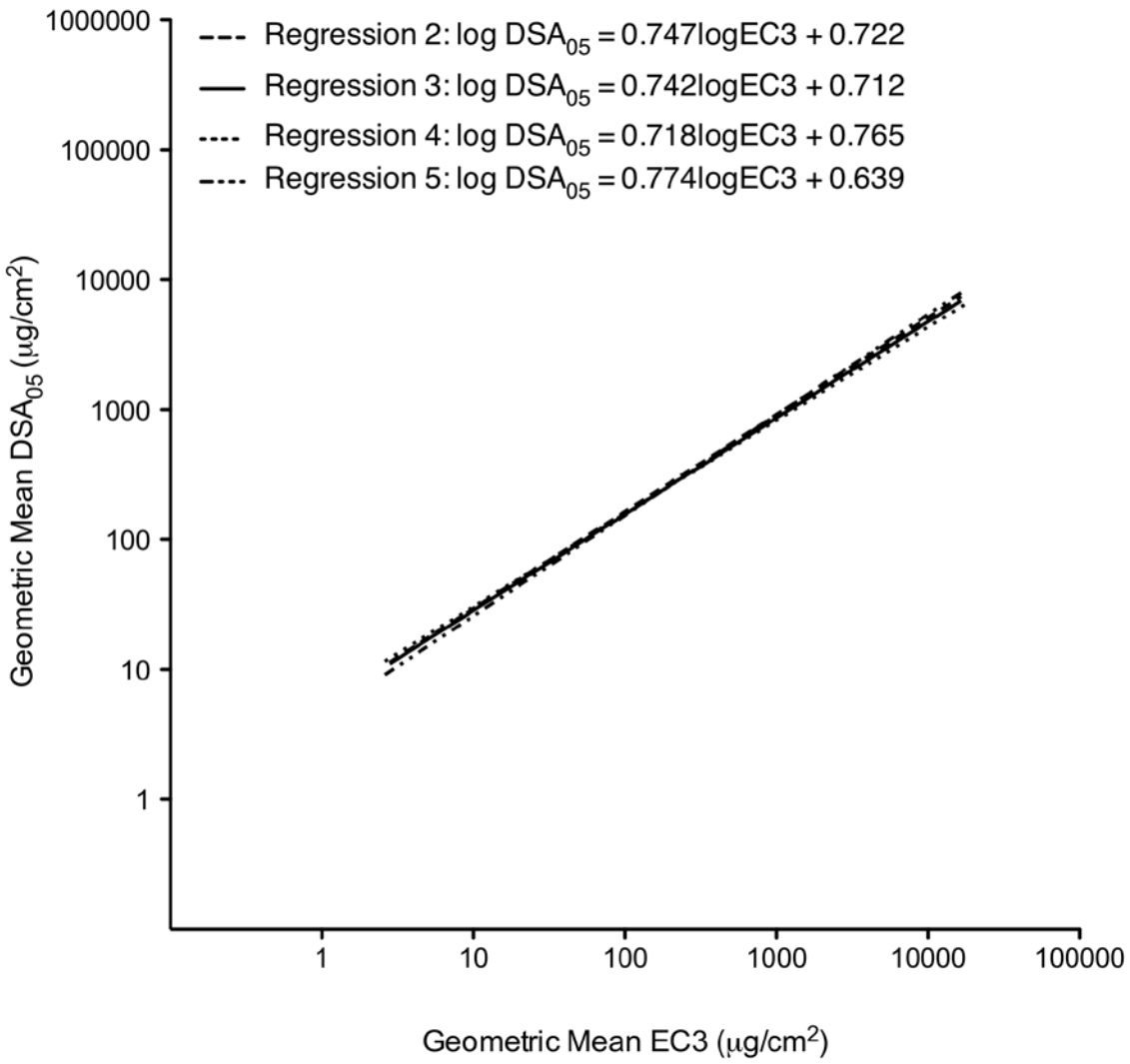
Figure C-IV-1 Most Potent and Geometric Mean Linear Regressions for LLNA EC3 versus Human DSA_{05} for 63 LLNA and Human Skin Sensitizers



Abbreviations: DSA_{05} = induction dose per skin area, in $\mu g/cm^2$, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, which is the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay.

The regressions correspond to regressions 1 and 2 in **Table C-IV-2**. The triangles and solid line show the data and regression line for the most potent EC3 value versus the corresponding human DSA_{05} (both in $\mu g/cm^2$) for 63 sensitizers. The circles and dashed line show the data and regression line for the geometric mean EC3 versus the corresponding geometric mean human DSA_{05} for the same substances. The geometric mean value was used for substances with more than one value. Geometric mean calculations of the EC3 excluded discordant negative results and ignored vehicle (i.e., results for all vehicles were pooled).

Figure C-IV-2 Geometric Mean Linear Regressions for LLNA EC3 versus Human DSA_{05} for 63 LLNA and Human Skin Sensitizers



Abbreviations: DSA_{05} = induction dose per skin area, in $\mu g/cm^2$, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, which is the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay.

The regressions of EC3 versus the corresponding human DSA_{05} (both in $\mu g/cm^2$) for 63 sensitizers correspond to regressions 2, 3, 4, and 5 in **Table C-IV-2**. So that the regressions can be viewed with more clarity, the data points, which are very similar for each regression, are not shown.

assigning an EC3 value of 110%) or exclusion of discordant negative tests had no noticeable impact on the regression analyses. All Spearman correlations for regressions 2, 3, 4, and 5 were highly significant ($p < 0.0001$). Correlations 2 and 3 had the highest correlation coefficient, $r = 0.692$.

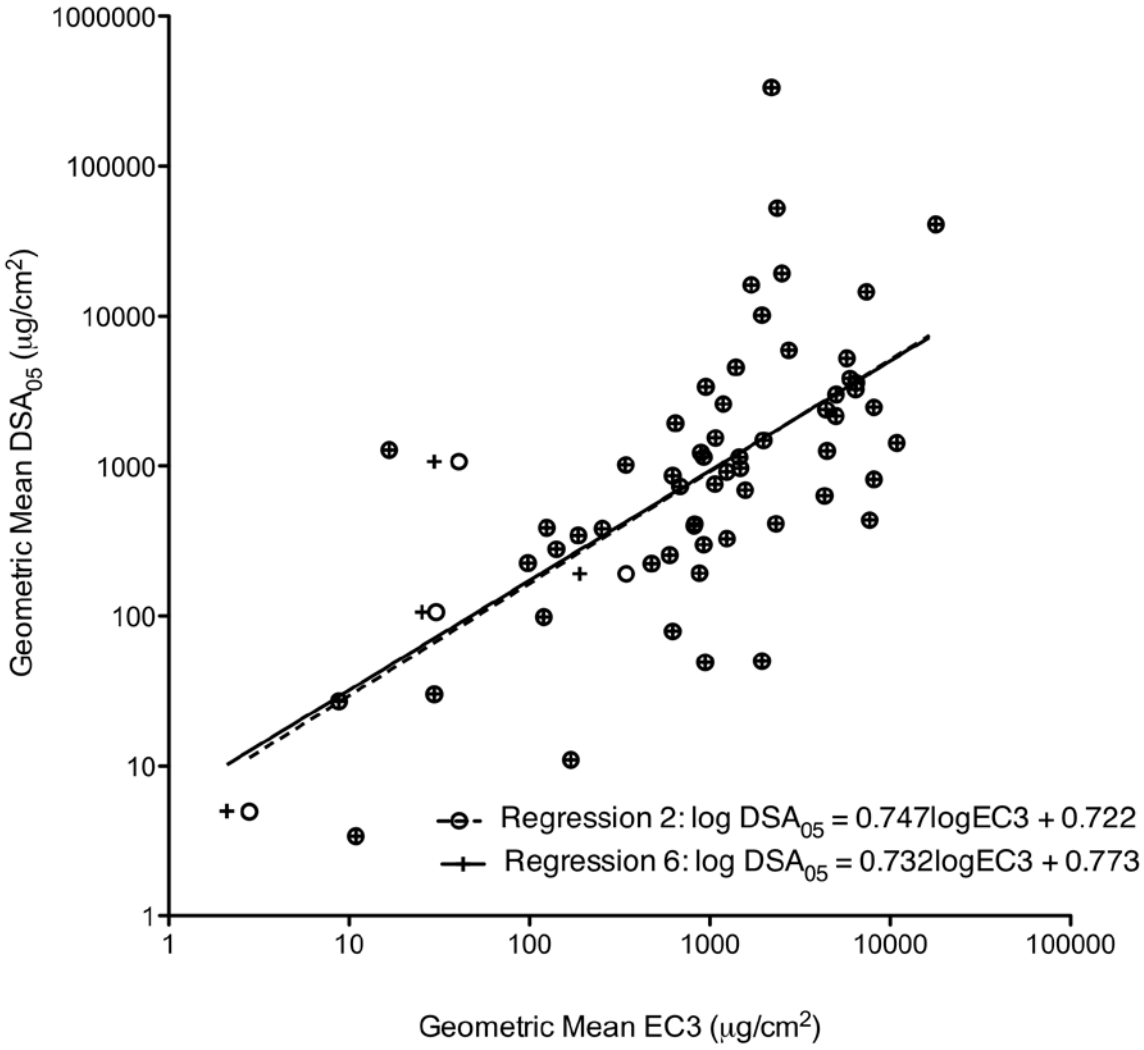
The multiple linear regression analyses detailed in **Table C-IV-2** were performed to determine the optimum approach to apply in subsequent analyses (i.e., the calculation of correct, under-, and over-classification of human potency category by the EC3 value in **Sections 6.1** and **6.2** of the BRD). All of the geometric mean regressions yielded higher R^2 values than the regression that used the most potent values (**Table C-IV-2**). Because the geometric mean regressions (2, 3, 4, and 5) produced very similar results (**Table C-IV-2**; **Figure C-IV-2**) the optimum approach was considered to be the simplest computational approach, regression 3.

2.4.2 Confirming the ANOVA Results

To confirm the two-way ANOVA results reported in **Sections 2.1** and **2.3**, two additional regressions were performed. These regressions used the same approach as regression 2 for combining LLNA results: discordant negative results and vehicles were ignored in calculating the geometric mean EC3. Regression 6 confirms the two-way ANOVA results in **Section 2.1** that indicated that vehicle was not an important determinant of EC3 value in the current database (**Table C-IV-2**). When LLNA results that used propylene glycol (seven tests for the substances in the regression) and Pluronic L92 (15 tests for the substances used in the regression) were excluded from the analyses, regression 6 was similar to regression 2, which included these tests (**Figure C-IV-3**). For regressions 2 and 6, the standard errors for the slopes and intercepts easily overlapped (**Table C-IV-2)**. Thus, the use of multiple LLNA vehicles in deriving the geometric mean EC3 value was confirmed to have no significant effect on the regression results.

Regression 7 was performed to confirm that the use of EC3 values from standard, nonstandard, and unreported protocols would not significantly affect the EC3 versus DSA_{05} regression (**Table C-IV-2**). The two-way ANOVA reported in **Section 2.3** indicated that protocol was not a significant determinant of EC3 value. Regression 7 was performed with only the LLNA results that were generated using standard protocols (Dean et al. 2001; ICCVAM 1999; OECD 2002). Excluding the LLNA tests from nonstandard or unreported protocols reduced the total number of LLNA tests from 375 (for 63 substances) included in regression 2 to 261 (for 54 substances) in regression 7. Nine substances were excluded from regression 7 because they had no positive LLNA tests using a standard protocol. Even with the exclusion of 30% of the LLNA results, regression 7 is similar to regression 2 (**Figure C-IV-4**). The standard errors for the slopes and intercepts easily overlap. Regression 2, however, is the preferred regression because it uses more data and also has a higher R^2.

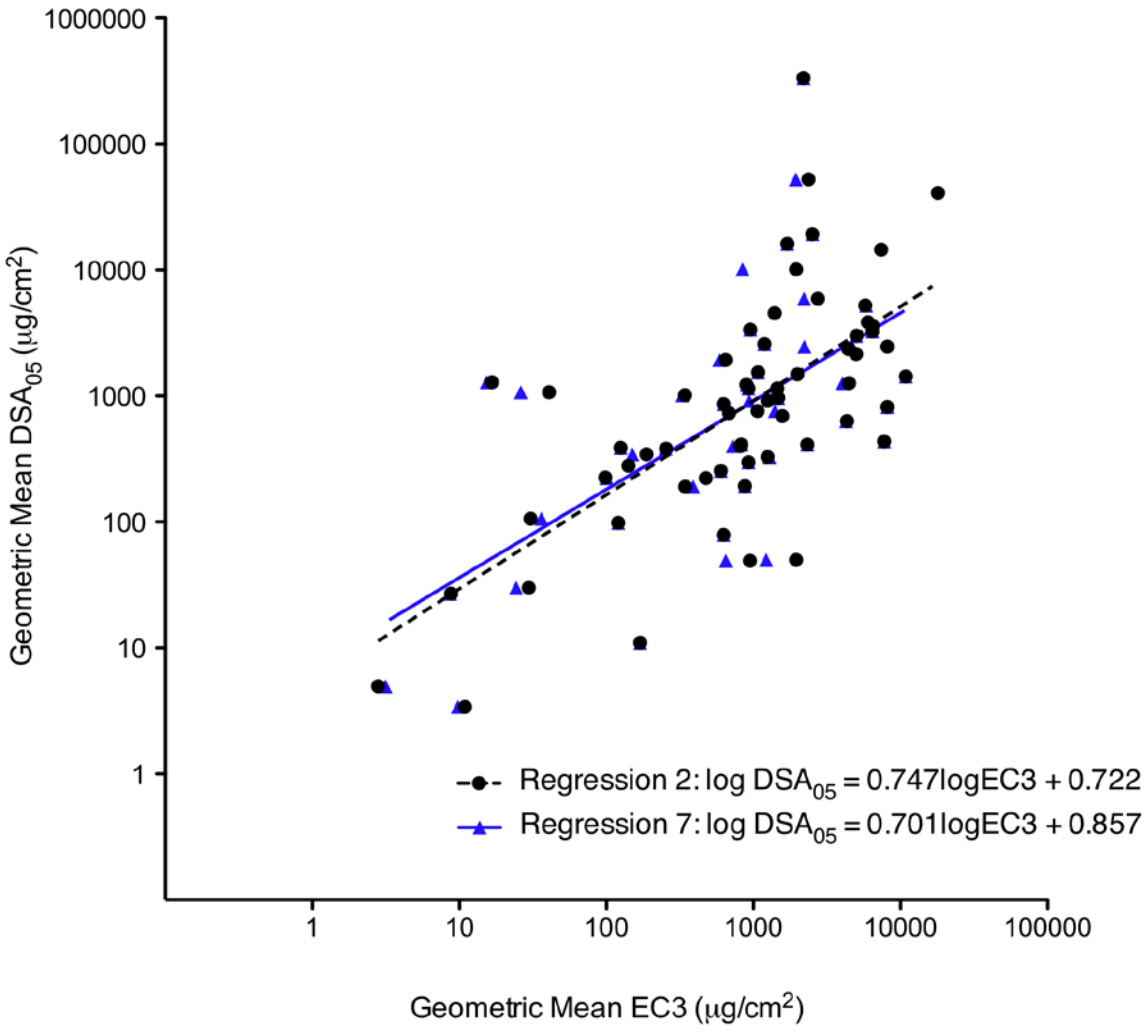
Figure C-IV-3 Geometric Mean Linear Regressions for LLNA EC3 versus Human DSA_{05} for 63 LLNA and Human Skin Sensitizers With or Without Propylene Glycol and Pluronic L92 Tests



Abbreviations: DSA_{05} = induction dose per skin area, in μg/cm², in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, which is the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay.

The regressions correspond to regressions 2 and 6 in **Table C-IV-2**. Both regressions show the geometric mean EC3 value versus the corresponding geometric mean human DSA_{05} (both in μg/cm²). The EC3 values for the circles and dashed line regression (2) include LLNA tests that use propylene glycol and Pluronic L92 as vehicles. The EC3 values shown by the diamonds and solid line regression (6) exclude tests that use propylene glycol and Pluronic L92 as vehicles. Many of the data points for the two regressions are coincident.

Figure C-IV-4 Geometric Mean Linear Regressions for LLNA EC3 versus Human DSA_{05} Using Standard (n = 54) or Standard and Nonstandard LLNA Protocols (n = 63)



Abbreviations: DSA_{05} = induction dose per skin area, in µg/cm^2, in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population; EC3 = estimated concentration of a substance expected to produce a stimulation index of 3, which is the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay.

The regressions correspond to regressions 2 and 7 in **Table C-IV-2**. Both regressions show the geometric mean EC3 value versus the corresponding geometric mean human DSA_{05} (both in µg/cm^2). The EC3 values for the circles and dashed line regression (2) include LLNA tests from both standard and nonstandard protocols. The EC3 values shown by the triangles and solid line regression (7) include only LLNA tests that use the standard LLNA protocol (Dean et al. 2001; ICCVAM 1999; OECD 2002).

3.0 References

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