

ICCVAM EXPERT PANEL REVIEW OF IN VITRO TEST METHODS FOR IDENTIFYING OCULAR CORROSIVES AND SEVERE IRRITANTS

Public comment concerning the HET-CAM Background Review Document (BRD)

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Background of the HET-CAM validation study in Germany

At the time, when the validation study of the HET-CAM test was started in Germany, in 1987, no scientific concept had been developed for the experimental validation of in vitro toxicity tests. At the first international workshop on the validation of in vitro toxicity tests held in Amden (Switzerland) in 1990, I was able to give major input from the experience of the German HET-CAM validation trial. After the recommendations of this workshop on the concept how to conduct validation studies, which were published in 1990 (Balls et al, 1990), we tried to incorporate these principles into the German HET-CAM validation study. In an early publication on the *“interlaboratory assessment of alternatives to the Draize eye irritation test in Germany”* (Spielmann et al, 1991) we have therefore outlined in the introduction that *“the validation project consist of the following three parts as suggested by a recent CAAT/ERGAAT workshop (Balls et al. 1990): (i) a preliminary phase, (ii) an interlaboratory assessment, (iii) the development of a database of results.”*

Taking into account the failure of the Amden concept (Balls et al., 1990) for the validation of toxicity tests, in 1995 two new essential elements were added, the prevalidation phase and the incorporation of biostatistically based prediction models (PMs)/data interpretation procedures (DIPs) (Balls et al. 1995). The new concept for the validation of toxicity tests was internationally accepted at the OECD level in 1996 (OECD, 1996) and it is based on the ICCVAM and ECVAM principles for the validation of toxicity tests

We have tried to implement the new validation principles into our on-going validation study of the HET-CAM test in the following manner:

1. Establishing the HET-CAM test in laboratories participating in the validation study

In the “preliminary phase” of our study (Kalweit et al., 1987; Kalweit et al. 1990) the HET.-CAM test was established in the participating laboratories. Intra- and inter-

laboratory reproducibility was determined with five test chemicals (2-butoxyethanol, dimethylsulphoxide (DMSO), triethanolamine, SDS and zinc pyridinethione). We have published the results in 1990 (Kalweit et al. 1990) and we have indicated in the results section (pg 703) "***that at this stage of the validation study, a comparison of the data from the two in vitro tests (the second one is the 3T3 NRU cytotoxicity test) with in vivo data from the Draize rabbit eye test or even human data is not possible.***"

The inter-laboratory reproducibility in eight laboratories is shown for the endpoint irritation score (IS) for two test chemicals in Figure 1 (butoxyethanol) and in Figure 2 (dimethylsulfoxide). **According to our evaluation, the interlaboratory reproducibility of the HET-CAM test is quite satisfactory for the two chemicals.** To assess the predictive value of the HET-CAM test, classifications obtained with the five test chemicals are compared to the classification according to EU standards for existing chemicals in 1990 in Table 1. The table shows that the in vivo corrosive test chemical induced the highest IS score in the HET CAM test and that the chemical, which induced only a slight reaction in the rabbit eye also induced the lowest increase in the IS score.

Conclusion: The inter-laboratory reproducibility of the HET-CAM test was shown with five test chemicals in eight laboratories and the severity of reaction in the HET-CAM test corresponded to the irritating effects of the substances in the Draize rabbit eye test..

2. Inter-laboratory reproducibility

In our second publication ([Spielmann et al., 1991](#)) we reported the results of the "interlaboratory assessment" stage of the HET-CAM test validation study (*and of the 3T3 NRU cytotoxicity tests*) in 12 laboratories. In the statistics section we reported that the estimates for the interlaboratory reproducibility was calculated according to the recommendations of the International Standard Organization (ISO 5725) for the 3T3NRU test. To assess the interlaboratory reproducibility of the HET-CAM test in the same study, the classification results of individual laboratories were compared, using the Irritation Score (IS) as described in the INVITOX/ECVAM protocol (Spielmann and Liebsch, INVITTOX 1992).

The "interlaboratory assessment phase" was conducted with 10% solution of coded chemicals. Since 5 out of the 32 chemicals, which could not be tested as 10% solutions in either water or oil due to low solubility, interlaboratory variability could only be deter-

mined for 27 out of the 32 chemicals.

Moreover the “interlaboratory assessment” stage also served to improve the prediction model (IS score) and the preliminary test protocol, which was finalized by the end of this stage. As a consequence, there was a considerable variability in the results obtained with the preliminary HET-CAM protocol. Since it was the goal to classify chemicals for their eye irritation potential according to HET-CAM results, in Table 2 of the publication by Spielmann et al. 1991 an IS score of 10 is used to discriminate between severely irritating and non irritating chemicals. Since not all of the laboratories provided results for each test chemical, in some cases data are only given for 11 chemicals. Table 2 shows that the results obtained with the preliminary protocol were reproducible at the lower and upper end of the Draize eye irritation scale while there was a considerable variability in the medium range in a similar manner as in the Draize rabbit eye test (Weil and Scala, 1971; Balls et al. 1995).

Another important aspect of the “interlaboratory assessment” stage of the study was that for animal welfare reasons no Draize eye tests were conducted in rabbits and existing chemicals were chosen as test chemicals, for which Draize eye test data were available in the files of the Federal Health Office BfR, which is termed BfR today. Thus, the HET-CAM data in this early stage can only be compared to the classification that were used for regulatory purposes in the EU in 1991.

Conclusion: The second stage of the German HET-CAM validation study (Spielmann et al. 1991) was conducted as an “interlaboratory assessment” to determine the interlaboratory reproducibility of the test and to improve the test protocol. No Draize rabbit eye tests were performed and high quality data from the files of BfR were used to assess the predictive value of the HET-CAM test.

3. Development of a HET-CAM data base

It was the goal of the third stage of the German validation study of the HET-CAM test “to develop a data base“ of up to 200 chemicals according to the recommendations of the Amden workshop (Balls et al. 1990, see *section 1*). We have, therefore, tested 136 chemicals provided by participating companies of the German chemical industry in the HET-CAM test. The companies also provided the Draize rabbit eye test results for each of the chemicals. In order to test 136 chemicals within an acceptable time frame and

since the reproducibility had been established previously (Spielmann et al., 1991, see #2), it was decided to test each chemical coded under blind conditions in two laboratories.

In the first short publication of the third stage of the study (Spielmann et al., 1993), the data base development stage, we have classified the 136 chemicals according to their HET-CAM data by applying an empirically derived prediction model, in which the Irritation Score (IS) and the Irritation Threshold Concentration (ITC) were combined. The results for the 136 chemicals are summarized in Table 2, in which differences in the classification results by the two laboratories are described in detail. It has to be taken into account that at the time, when the validation study was conducted, the classification criteria of the Draize eye test for severely eye irritating chemicals (R 41) were changed to include also mildly and moderately irritating chemicals, which are inducing irreversible damage. Therefore, to the R-41 classification group (Group 5) we have added two subgroups of moderate or mild irritating chemicals, that are inducing irreversible during a 21 day period after treatment have been added (Group 4 + Group 3). In this short publication an overall evaluation of the results of the “database development” stage is given.

Conclusion: Since this publication (Spielmann et al., 1993) only served to give a short summary of the results obtained with 136 chemicals in the “the database development” stage, no individual data of the results of the HET-CAM test or of the corresponding Draize eye test data are given.

4. Detailed analysis of 200 chemicals tested in the HET-CAM test

A detailed analysis of all of the data obtained with 200 chemicals in all stages of the German validation study of the HET-CAM test was published in 1996 (Spielmann et al., 1996; 117 pages!). The most important element of the publication, the background data used in the biostatistical analysis is documented in the 7 Appendixes (I – VII). To facilitate the review process we have in June of 2004 submitted upon request of NICEATM an MS.EXCEL file with the complete data set that was used in the analysis of our publication to NICEATM. ***Although NICEATM has acknowledged the receipt of this MS.EXCEL data file (entitled ATLA96-annexes.xls) in July of 2004, this important document is not mentioned in the HET-CAM BRD and it is missing in the list of references.***

Thus, from the scientific point of view an important document of the German validation study is missing in the HET-CAM BRD. Moreover, the way in which the results are reported is not correct. I am now referring to chapter 5.0 of the HET-CAM BRD entitled "HET-CAM test method data and results". In section 5.4.8 of the HET-CAM BRD our publication (Spielmann et al., 1996) is evaluated in the following manner (pg. 5-13, lines 358-361): "***In this evaluation of the HET-CAM test method, 118 test substances were evaluated in one laboratory. HET-CAM test method data on the 118 substances were included in the published report as were the corresponding ocular irritancy classification for each substance. Detailed in vivo data were not available for test substances, however classifications according to EU.***"

In contrast to this statement the second paragraph of the summary of our publication (Spielmann et al., 1996) reads as follows: .. ,a 2-year database development was conducted as Phase II, during which 166 code chemicals were tested in the two in vitro tests (HET-CAM and 3T3 NRU cytotoxicity test), each of them in two laboratories. Test chemicals backed by high-quality Draize eye test data were provided by industry and selected to represent a wide spectrum of chemical classes and eye irritation properties. Independent quality control of in vitro and in vivo data and biostatistical evaluation were performed during an additional BMBF-project on biostatistics. In the quality assurance step, which is an essential step in biostatistics, the number of chemicals was reduced to 143, and these data were entered into an MS.EXCEL file "ATLA96-annexes.xls" to facilitate determination of in vitro/in vivo correlations."

In contrast to the statement in the HET-CAM BRD you will find the data obtained in the two laboratories with the HET-CAM test in the MS.EXCEL file "ATLA96-annexes.xls" and in Appendix II of the publication Spielmann et al., 1996, pg. 800-820: "Appendix IIa Results obtained with the HET-CAM test and the physicochemical properties of the test chemicals for laboratory I" (pg. 800-810) " and "Appendix IIb: Results obtained with the HET-CAM test and the physicochemical properties of the test chemicals for laboratory I" (pg. 811-819)". For your information I have enclosed the MS.EXCEL file "ATLA96-annexes.xls".

Moreover, in contrast to the statement in the HET-CAM BRD you will find the data

obtained in vivo in the Draize eye test in vivo in the MS.EXCEL file "ATLA96-annexes.xls" and in Appendix IV of the publication Spielmann et al., 1996, pg. 834-847. In particular, you will find in Appendix IV detailed information for each chemical on the conjunctiva (erythema and chemosis), on the iris and on the cornea for time points from 1hour up to 72 hrs. There are, of course, no Draize eye test data for the existing chemicals in these lists, since they were tested in the "interlaboratory assessment stage" and the Draize eye test classification data were taken from the files of the BGA (Spielmann et al., 1993; see #3). Moreover, the Draize eye test data are given as the means of the 3 rabbits rather than as individual data for each rabbit, since we found this information sufficient for our classification purposes.

Recommendation: The MS.EXCEL file "ATLA96-annexes.xls" should be added to the official list of references provided to the reviewers by NICETAM and it should be evaluated in the HET-CAM BRD.

5. **Individual Draize eye test data for each rabbit.**

In order to facilitate the evaluation of the HET-CAM data we are providing you with an additional MS.EXCEL file entitled "GermanHetCamStudy.zip", in which you will find the individual data for each rabbit for the chemicals that are also given in Appendix IV of our publication (Spielmann et al., 1996; see above #4). In this data file the Draize eye test data are recorded for up to 21 days.

Recommendation: The MS.EXCEL file "GermanHetCamStudy.zip" should be added to the official list of references provided to the reviewers by NICETAM and it should be evaluated in the HET-CAM BRD.

6. **Endpoints recorded in the HET-CAM test.**

In the extensive publication of the German HET-CAM validation study (Spielmann et al, 1996) we have reported **that nine endpoints were determined in the HET-CAM test and used in discriminant analysis to identify the most predictive endpoints in the HET-CAM test to identify severely eye irritating properties of test chemicals. The 9 endpoints are given on pg. 764 in section 2.2.2.1 "Endpoints (HET-CAM test and 3T3 NRU cytotoxicity test) used in the discriminant analysis."** The endpoints that were recorded during the validation study are given in the MS-EXCEL file and in Appendix VII of

our publication (pg. 853-858). These endpoints are given as means of the values determined in two laboratories (see above #5) and they were used in the development of a prediction model to identify severely eye irritating chemicals.

Recommendation: NICETAM should evaluate the nine missing endpoints of the HET-CAM and include it in the HET-CAM BRD.

7. Development of a prediction model to identify severely eye irritating chemicals using the HET-CAM endpoint mtc100 “mean detection time for appearance of coagulation when using a 100% solution”

The biostatistical data are given in our publication Spielmann et al. 1996 in sections 2.2.3.3.4, 2.2.3.3.5 and 2.2.3.3.6 on pg. 774-778). The data analysis described in this section of the publication and Figures 10,11 and 12 clearly indicate that the endpoint mtc100 (defined on pg. 764 as “mean detection time for appearance of coagulation when using a 100% solution”) provides a very simple means to identify severely eye irritating chemicals, since all chemicals characterized by a mtc100 of <100 seconds are severely irritating.

The rate of false positives results obtained when applying this prediction model of the HET-CAM test with the whole set of 200 test chemicals was 0 ! To act even more on the safe side and as a general rule we propose to use a mtc100 of <1min (or 60 seconds) to classify severely eye irritating chemicals in the HET-CAM test.

Conclusion & recommendation: The data analysis of our study proves that no further testing in vitro or in vivo is required, if an mtc100 of <1min is determined in the HET-CAM test. NICETAM should evaluate the prediction model based on the endpoint mtc100 and include it in the HET-CAM BRD, since this will allow to considerably reduce testing severely eye irritating materials in the Draize rabbit eye test.

8. Testing of chemicals insoluble in water, solvents and insoluble materials: In the “database development “stage insoluble and soluble materials were tested successfully. The details on physicochemical properties and solubility are given for each chemical in the MS-EXCEL file and also in Appendix IIa and IIb of our publication (Spielmann et al. 1996, pg. 800-820). In addition, the solvent used is indicated and for solid materials even the exposure time (1min or 5 min).

Conclusion + recommendation: In the HET-CAM BRD this important information is ignored. It should be evaluated by NICETAM and be included in the HET-CAM BRD.

9. Publications missing in the literature provided with the BRDs

9.1 "IRAG Working group 2 CAM-based assays" by Spielmann et al., 1997, Food and Chemical Toxicology 35, 39-66.

In 1993 the US Interagency Regulatory Alternatives Group (IRAG) held a workshop on "Eye irritation testing; practical applications of non-whole animal alternatives". For several in vitro alternatives, which are currently evaluated by the ICCVAM expert panel review, extensive analysis of the in vivo/in vitro correlations have been assessed. I wonder why the NICEATM expert group did not provide the expert reviewers panel with these documents but only mentioned them in the list of references. The one to which I have contributed may be helpful for the experts working on the HET-CEM BRD. Moreover, this activity was sponsored by several of the Federal US agencies, which stakeholders of ICCVAM today.

Recommendation: The publication should be added to the official list of references provided to the reviewers by NICETAM and it should be evaluated in the HET-CAM BRD.

9.2 Journal Officiel de la Republique Francaise dated 29 decembre 1996, pg. 19137-19138 (Official Journal of the French Republic December 29, 1996, pg. 19137-19138) "Arrete du 29 novembre 1996 relatif aux methodes officielles d'analyse necessaires aux contoles des produit cosmetiques"

In this document of the "Federal Register" of the French Republic a new Annex IV to the French cosmetics directive has been published, in which the "hens egg chorion-allantoic membrane test" is accepted as an official test guideline for the safety testing of cosmetics for regulatory purposes. Thus, the HET-CAM test is officially accepted for regulatory testing in one of the EU and OECD member states.

This important piece of information is given in the HET-CAM BRD, although I have provided NICETAM with a copy of the document, as you can see from the attached copy of my letter to NICEATM dated July 9, 2004 ([copy attached as PDF file entitled "HSp BRD letter 09-07-2004.pdf"](#)). For your information I am attaching the MS.WORD file entitled "[French_Guidline_eye_irritation.pdf](#)", which contains a copy of the 2 pages from the Offi-

cial Journal of the French republic and a cover letter drafted by the French association of the perfume industry dated 15.01.97.

Recommendation: The publication should be added to the official list of references provided to the reviewers by NICETAM and it should be evaluated in the HET-CAM BRD.