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Ihre Zeichen und Nachrichten vom	Gesch.-Z.: Bitte bei Antwort angeben	Tel.-	Datum	Org.-Einheit/Ansprechpartner
July 25, 2005	AL 3 - 4711/FG 37-2005	fone 2270 fax 2958	12.09.2005	Head of Department 3 & Head of ZEBET

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

Public comment concerning the "Accuracy and Reliability Reanalysis Addendum" - Executive Summary and Section IV: HET-CAM Test Method, dated 25 July 2005

Dear Dr. Stokes,

as you know I am serving as an expert for the IRE (Isolated Rabbit Eye) test on the ICCVAM expert panel of in vitro methods for identifying ocular corrosives and severe irritants and as a reviewer for the BCOP test.

To support the ICCVAM Expert Panel Review I have submitted several data sets for review that were generated in a national German validation study of the HET-CAM test.


Due to my involvement in organizing the "5th World Congress on Alternatives and Animal Use in the Life Sciences" that was held in Berlin two weeks ago, I have been able only the new IRE and BCOP data sufficiently but not the analysis of the HET-CAM data that we submitted.

However, after studying the HET-CAM BRD and taking into account my responsibility as principal investigator and author of several of the documents that I submitted to NICEATM, I want to comment on the reanalysis of the HET-CAM data, since some of the information that I provided is not documented appropriately both in the executive summary and in Section IV, which is covering the HET-CAM test. Moreover, in several instances the authors of SECTION IV do not refer to the correct literature or have ignored and not discussed important information published previously.

Taking into account the extensive biostatistical evaluation of the original validation study of the HET-CAM test in Germany (Spielmann et al., 1996), which was conducted in a joint national biostatistical project funded by the Federal German Minister for Research and Technology (BMBF) I want to ask you to provide the experts of NICEATM and the expert panel of the HET-CAM BRD with the enclosed information, which may help to better evaluate the HET-CAM documents that Dr. Manfred Liebsch and I have submitted.

Please excuse again that I did not manage to send our comments any earlier. I do however hope, that the expert panel will take our comments into account before finalizing the BRD and the "*Accuracy and Reliability Reanalysis Addendum*".

With the best regards
Sincerely



Dr. med. Horst Spielmann
Direktor und Professor
Head of Dept. 3 "Scientific Services"
and Head of ZEBET

Enclosures: public comment with 5 attachments

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Public comment concerning the "Accuracy and Reliability Reanalysis Addendum" - Executive Summary and Section IV: HET-CAM Test Method, dated 25 July 2005

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by Horst Spielmann (BfR, Berlin, Germany)

My colleagues and I at the BfR, the Federal Institute for Risk Assessment in Berlin, Germany, have in particular reviewed SECTION IV, since we have submitted HET-CAM data for this part of the document. Moreover, I have previously on December 30, 2004, submitted a detailed public comment to the data published in the "HET-CAM Background Review Document (BRD)".

My colleagues and I are pleased that the expert panel and the NICEATM staff has taken most of our comments and recommendations into consideration. However, some of our comments and recommendations, have so far not been evaluated. On behalf of the consortium of more than 12 laboratories that conducted the validation study of the HET-CAM test in Germany and on behalf of the Federal Minister for research and technology, which has funded the study, I want to ask both the members of the expert panel and the staff of NICEATM to take our public comment into consideration before finalizing the BRG and the "Accuracy and Reliability Reanalysis Addendum".

To facilitate the discussion, we are addressing the various points separately.

1. Reference Kalweit et al. 1987, please change to or to Kalweit et al. 1989 or 1990 or to ECVAM-SIS INVITTOX Method #47.

In the executive summary, e.g. in the footnote to Table ES-1, and in the list of references in SECTION IV you are referring to the reference Kalweit et al. 1987. However, in all existing reference databases you will find that the reference Kalweit et al. 1987 does not exist. Dr. Kalweit joined our group in 1988, when the German validation study started. The correct year for this reference is 1989 as you can see from the attached copy of page 1 of the publication ([attachment #1](#)).

In the BRD you have used the correct reference, Kalweit et al. 1989. However, the "Journal of Molecular Toxicology" is not a peer reviewed journal and has only published a single volume in 1989, vol. 1, in which the manuscript was published.

From our perspective it is more appropriate to refer to the first publication in a peer reviewed journal. We therefore want to suggest to use the reference "Kalweit et al., Toxicology in Vitro 4, 720-706, 1990".

2. Correct reference of the IS(B) method

To assess irritation in the HET-CAM test usually the Irritation Score (IS) is used. In the review documents you are referring to two ways of calculating the IS, IS(A) and IS(B):

- a) IS(A) is the approach first described by Luepke (1985). IS(A) is calculated from three reactions on the CAM injection (i), haemorrhage (h) and coagulation.
- b) IS(B), which we have used in validation study of the HET-CAM test in Germany, is not taking into account the same endpoints as IS(A) for the following reason:
The endpoint "injection" could not reproducibly be determined in the laboratories participating in the German validation study. Therefore, we have used the three reactions (endpoints) on the CAM haemorrhage (h), lysis of vessels (l) and coagulation (c).

To calculate IS(B), the CAM is observed for a period of 5 min (300 seconds) and the time is recorded, when each reaction is observed on the CAM for the first time. The three reaction times for h, l and c are used to calculate IS(B).

We have first described this method in the publication by Kaltweit et al. 1989. However, for the reasons described above, we suggest to use the reference Kalweit et al. 1990 and the ECVAM-SIS INVITTOX Protocol #47 (IP-47) (attachment #2), which was first published by our group in 1992 and which is currently available on the internet (<http://ecvam-sis.jrc.it/invittox/static/invittox.html>). Moreover, we do wonder, why you are not referring to the established INVITTOX Protocol #47 (IP-47) in the BRD and in SECTION IV of the "Addendum".

Recommendation to #1 & #2:

Rather than referring to the reference Kalweit et al. 1987 for the IS(B) method in the executive summary, e.g. in the footnote to Table ES-1, and in the list of references of SECTION IV, I want to ask you to use the correct references, which underwent a peer review process as described above Kalweit et al. 1990 and ECVAM-SIS INVITTOX Protocol #47 (IP-47) (<http://ecvam-sis.jrc.it/invittox/static/invittox.html>).

3. Differences between IS(A) and IS(B) methods for calculating the irritation potential in the HET-CAM test

As outlined above, Luepke (1985) used "injection" as one of the three endpoints for determining IS(A) to assess the irritation potential on the CAM, while we used "lysis of vessels" (l) instead to calculate IS(B). Therefore, the information provided by IS(A) and IS(B) is not identical. Moreover, the algorithms for calculating IS(A) and IS(B) are not taking into account identical time periods for determining the first appearance of the three reactions (endpoints) on the CAM.

Thus, the experts of NICEATM and of the ICCVAM Expert Panel have to ensure from the "materials and methods" section of each HET-CAM study, which of the prediction models IS(A) and IS(B) has been applied and, consequently take this information into account when evaluating the "*Accuracy and Reliability*" for each of the HET-CAM studies.

Recommendation #3:

Since it is not clear from the executive summary and from SECTION IV "HET-CAM test" that the specific differences between IS(A) and IS(B) have been taken into account in the evaluation by the NICEATM team, this important detail of the HET-CAM test should be reevaluated before the report is finalized.

4. Evaluation of the classification of test chemicals in the HET-CAM test

Both in the ECVAM-SIS INVITTOX Protocol #47 (IP-47) and in our publications Spielmann et al. 1993 and 1996, we have described the prediction model that was used in the German validation study of the HET-CAM test, to classify test chemicals according to their eye irritation potential on the eye. This prediction model takes into account both the IS(B) and the "threshold concentration" (TH). Thus, in the German validation study of the HET-CAM test, the IS(B) was never used as prediction model for classifying severely irritating test materials.

In their evaluation given in the *"Accuracy and Reliability Reanalysis Addendum: HET-CAM Test Method - SECTION IV"* the authors do not explain, why they have only used the IS(B) for classifying chemicals tested in the German HET-CAM test validation study and not the prediction model used in the German validation study as described both in the ECVAM-SIS INVITTOX method #47 and in the publications by the German consortium (Spielmann et al. 1993 and 1996):

Recommendation #4:

It must be discussed in the *"Accuracy and Reliability Reanalysis Addendum"*, why the authors did not consider to use the prediction model used in the German validation study of the HET-CAM test, which is also the current prediction model in the ECVAM-SIS INVITTOX Protocol #47 (IP-47).

5. Correction to Table IV-8 in the Addendum to the In Vitro ocular Toxicity Draft BRD

The content of Table VI-8 is discussed in Section IV on pg. IV-25-IV-27 under the heading *"2.4 Accuracy of the HET-CAM IS(B) Analysis Method for the GHS Ocular Hazard Classification System by Chemical Class and Property of Interest"*. Taking into account the number of chemicals evaluated in Table IV-8, we do assume that the data were taken from our publications Spielmann et al., 1993 and 1996. However, the authors are not giving reference to any publication in chapter 2.4.

Moreover, since we have tested chemicals both in 10% and 100% solutions and since the numbers of chemicals analyzed is in the range of the numbers of chemicals tested in the German validation study, we do assume that the classifications are based on our results.

As outlined above, the prediction model for classifying test chemicals in the German validation study is not based on the IS(B), which was used by the NICEATM. The BGA prediction model for classifying test chemicals according to their eye irritation potential Table II on pg. 761 of our publication Spielmann et al. ATLA 24, 741-858, 1996. The classification results obtained in the German validation study of the HET-CAM test are given in Table III on the same pg. 761 of the same publication. The authors of the *"Accuracy and Reliability Reanalysis Addendum"* do not report our results and they do not discuss them in comparison to their classification results.

From the scientific point of view, we find it unacceptable that this point is not discussed throughout the *"Accuracy and Reliability Reanalysis Addendum"*.

Moreover, we are amazed about the classification results given on pg. IV-26 and IV-27. On pg. IV-26 the following results are given for false positive and false negative rates: For the 101 "overall IS(B)-10" chemicals and the 143 "over all IS(B)-110" chemicals false positive rates of 33 and 60% are given and false negative rates of 30% and 15% respectively. This

seems to be in contrast to the data given in the same table on pg. IV-27, since for 40 chemicals of "category 1 subgroup IS(B)-10" a false positive rate of 0% is reported and for 37 chemicals of "category 1 subgroup IS(B)-100" again the false positive rate is 0%.

Recommendation #5:

Since chapter 2.4 in SECTION IV is not meeting the scientific standard of a peer reviewed publication it has to be redrafted before it can be recommended for publication.

6. Reproducibility of classification results in the HET-CAM test

The first stage of the validation study of the HET-CAM test was conducted as an inter-laboratory validation study. The first short publication of this part of the study is entitled "Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany" (Spielmann et al, Toxic. In Vitro 5, 539-542, 1991) (attachment #4). Table 2 of this publication is entitled "A comparison of the results of the HET-CAM test carried out in 12 laboratories and the in vivo irritation potential (as assessed in the Draize eye test) for 27 chemicals". For your information I am attaching a copy of this publication.

Even when taking into account that the classification in the Draize eye test has slightly been changed according to harmonization of OECD Test Guidelines, the classification results given in table 2 for severely irritating and corrosive chemicals is impressive. According to the IS(B) scoring system a score >10 indicates severe eye irritation properties. According to the footnote to table 2, a substance was classified positive, when 75% of laboratories determined an IS(B) of >10. As you can see all of the corrosive chemicals were classified correctly and the majority of severely irritating test materials were also correctly classified.

This result is the main reason that regulators in Europe are accepting a positive results in the HET-CAM test for the classification of corrosive and severely irritating materials without any confirmatory testing in rabbits in vivo.

This important information has not been covered in the "Accuracy and Reliability Reanalysis Addendum". Since it is the goal of the joint ICCVAM/ECVAM exercise of reviewing existing data obtained in the four established in vitro alternatives to the Draize eye test, I have to ask the authors of the study and also the ICCVAM Expert Panel to comment on this important piece of evidence.

Recommendation #6:

It must be discussed in the "Accuracy and Reliability Reanalysis Addendum", why the authors did not report and discuss the reproducibility of the classification results for test chemicals, which are corrosive or severely irritating to the eye. In addition the consequences for testing these materials in vivo in rabbits should be explained.

7. References Spielmann H, Liebsch M 2005a and 2005b on pg. IV-88

The two references, which are given in Section IV of the "Accuracy and Reliability Reanalysis Addendum", are not correct for the following reasons:

We have in June of 2004 submitted to ICCVAM/NICETAM the publication in which the validation study of the HET-CAM test in Germany is described in detail (Spielmann et al. ATLA 24, 741-858, 1996) and we have also sent the background material as an "xls-file" to the scientists, who were responsible for the HET-CAM test at NICEATM. For your information I am attaching a copy of the e-mail, in which Dr. Neepa Choksi on July 8, 2004, confirmed that

she had received the electronic version of the German validation study. Attached to that e-mail is my e-mail dated June 14, 2004, in which I had sent the "xls-file" to Drs. Choksi, Stokes and Tice (attachment #4).

Thus, Dr. Manfred Liebsch and I did not submit "Unpublished data provided directly to NICEATM by H.Spielmann and M. Liebsch", as quoted in the references Spielmann H, Liebsch M 2005a and 2005b on pg IV-88.

Recommendation #7:

For the reasons explained, Dr. Manfred Liebsch and I would appreciate if the quotation in the two references is changed to "Unpublished data provided directly to NICEATM by H.Spielmann and M. Liebsch".

8. Publication missing in the literature provided with the BRDs and the Addendum

In my public comment dated December 30, 2004, to the BRD (attachment #5) I had clearly addressed that an important publication on the evaluation of different protocols of the HET-CAM test that was published under the auspices of IRAG, the Interagency Regulatory Alternatives Group, the predecessor of ICCVAM. My colleagues and I wonder, why this important publication has not been evaluated in the "Addendum SECTION IV". I am, therefore, repeating my request by copying the section from my earlier public comment to the BRD:

"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 are stakeholders of ICCVAM today.

Recommendation #8:

This publication should be added to the official list of references provided and it must be evaluated and commented on in SECTION IV of the "Addendum".

Betreff: German Validation Study Data and Fax
Von: "Choksi, Neepa (NIH/NIEHS)" <choksi@niehs.nih.gov>
Datum: Thu, 08 Jul 2004 17:10:12 -0400
An:" Horst Spielmann (E-mail)" <spielmann.zebet@bfr.bund.de>

Dear Horst --

We received your fax today regarding the HET-CAM BRD. Thank you so much for the additional information that you provided. We will review and include the information in the document.

I also wanted to thank you for the electronic version of the German Validation Study Data. I have been reviewing the information and the manuscript. I was hoping to ask you an additional question.

For the BRD, we would like to include the chemicals and the initial accuracy analysis in the 1996 validation paper (specifically, the analysis with the BGD prediction model). For that accuracy analysis, it is noted that 118 chemicals were evaluated using the BGA prediction model. I haven't been able to clarify which chemicals are included in this final set of 118 chemicals from the 152 new chemicals (which I have created from the information in the data you forwarded and the manuscript). Is there a list of which chemicals are included in the data set of 118?

I appreciate your help and comments.

All the best,
Neepa

Neepa Y. Choksi, Ph.D.
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> From: Zebet
> Sent: Monday, June 14, 2004 9:59 AM
> To: Choksi, Neepa (NIH/NIEHS); Stokes, William (NIH/NIEHS); Tice,
> Raymond (NIH/NIEHS); Haase, Manja
> Subject: Re: German Validation Study Data
>
> <<File: B1_9e1.xls>>

> Dear Neepa,
> please excuse that I could not get hold of the data any earlier. We
> contacted Dr. Moldenhauer, who worked on the data about 8 years ago.
> I am sending you all of the background data that were used in the German
> HET-CAM study in an excel-spread sheet, e.g. the Draize eye test data

> are in file B-4. Please look for details in the ATLA publication. I will
> return to my office on Friday June 18. If you have additional questions,
> please contact Manfred Liebsch, while I am gone.
> Best wishes
> Horst
>
> --
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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)

by Horst Spielmann (BfR, Berlin, Germany)

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 mct100 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.

Reference Articles

Attachment to Comment from Dr. med. Horst Spielmann, ZEBET
August 25, 2005

Kalweit A, Gerner I, Spielmann H. 1989. Validation Project of Alternatives for the Draize Eye Test. *Journal of Molecular Toxicology* 1:597-603.

Spielmann H, Gerner I, Kalweit S, Moog R, Wirnsberger T, Krauser K, Kreiling R, Kreuzer H, Luepke N-P, Miltenburger HG, Mueller N, Muermann P, Pape W, Siegemund B, Spengler J, Steiling W, Wiebel FJ. 1991. Interlaboratory Assessment of Alternatives to the Draize eye Irritation Test in Germany. *Toxic. In Vitro* 5(5/6):539-542.