

Preliminary Evaluation

Nomination of the Electrophilic Allergen Screening Assay for the Detection of Substances Causing Allergic Contact Dermatitis

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

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Testing for allergic contact dermatitis (ACD) hazard is an ICCVAM priority because it can result in significant unrelieved pain and distress to test animals, can involve large numbers of animals, and is required by multiple Federal agencies (ICCVAM 2008; ICCVAM 2012). ICCVAM evaluated the validation status of six new versions and applications of the murine local lymph node assay (LLNA) and finalized performance standards for modified versions of the LLNA. These modified test methods for ACD hazard reduce the number of animals required and eliminate the pain associated with a positive response compared with the traditional guinea pig tests, but do not, however, eliminate animal use. *In vitro* assays, which are undergoing development and validation, are expected to further reduce and eventually replace most animal use for ACD testing (ICCVAM 2012). These methods will be used to develop integrated testing and decision strategies to classify substances as sensitizers or nonsensitizers.

In June 2012, Dr. Paul Siegel, National Institute for Occupational Safety and Health, submitted a nomination to NICEATM for an *in chemico* test method for ACD hazard testing: the Electrophilic Allergen Screening Assay (EASA). The nomination requested that NICEATM and ICCVAM evaluate the test method as a screening assay for identifying contact allergens, and proposes collaborations with NICEATM to conduct validation studies and determine the most appropriate decision criteria to maximize the sensitivity and specificity of the assay. The priority of this nomination was evaluated by NICEATM based on the extent to which the following five ICCVAM prioritization criteria (ICCVAM 2003) apply to the EASA:

1. The extent to which the proposed test method is applicable to regulatory testing needs and agency programs
2. The potential for the proposed test method, compared to current test methods accepted by regulatory agencies, to:
 - Refine animal use (decrease or eliminate pain and distress)
 - Reduce animal use

- Replace animal use
- 3. The extent to which the proposed test method is warranted, based on the extent of expected use or application and impact on human, animal, or ecological health
- 4. The potential for the proposed test method to provide improved prediction of adverse health or environmental effects, compared to current test methods accepted by regulatory agencies
- 5. The extent to which the proposed test method provides other advantages (e.g., reduced cost and time to perform) compared to current test methods

Criteria 1: The extent to which the proposed test methods could be considered applicable to regulatory testing needs and agency programs

Due to the large number of animals used, the pain and distress that can result, and the requirements for ACD hazard information by several regulatory agencies, dermal toxicity testing, including ACD testing, is a high priority area for ICCVAM (ICCVAM 2008; ICCVAM 2012). The U.S. regulatory and public health agencies that have needs and/or requirements for ACD testing of substances and products include: Food and Drug Administration (FDA), Consumer Product Safety Commission (CPSC), Environmental Protection Agency (EPA), and Occupational Safety and Health Administration (OSHA). FDA regulations concerning the evaluation of safety for pharmaceuticals, which include the evaluation of ACD potential, are described in 21 CFR 312 (investigational new drug) and 21 CFR 314 (for marketing a new drug). CPSC requirements for ACD information are found in 16 CFR 1500.3. EPA requirements are found in 49 CFR Part 158, which details data requirements for pesticides, and in 49 CFR 700-799, which includes health effects testing requirements and hazard labeling per the Toxic Substances Control Act. OSHA does not require testing for their hazard communication standards in 29 CFR 1910.1200, but uses testing information to determine the appropriate precautionary labeling to protect workers.

Criteria 2: The potential for the proposed test methods, compared to current accepted test methods, to refine, reduce, or replace animal use

The primary test method to determine ACD hazards of most chemicals and products is the LLNA, which uses 20 animals per test substance when dose-response information is

required (EPA 2003; OECD 2010). The reduced LLNA, which can be performed when dose-response information is not needed, uses 12 animals per test substance (OECD 2010).

After penetration through the skin, the molecular initiating event in the adverse outcome pathway for skin sensitization is covalent binding of a hapten to protein in the skin (OECD 2012). The EASA consists of 2 simple chemical tests that assess the ability of electrophilic substances to bind to protein by measuring the depletion of free protein surrogates caused by covalent binding of the protein surrogate to the test substance. In the first test, depletion of 4-nitrobenzothiol (NBT), the protein surrogate for soft electrophiles, is measured by the loss of absorbance at 324 nm at 25°C for up to 2 hours. If depletion of NBT $\geq 30\%$, the test substance is classified as a sensitizer. If depletion of NBT $< 30\%$, confirmation tests must be performed. To confirm the result, the test substance is retested at twice the initial concentration of test substance and/or by increasing the reaction time to 4 hours. If depletion of NBT $< 30\%$, then the second test is performed. In the second test, depletion of pyridoxylamine (PDA), the protein surrogate for hard electrophiles, is measured by the loss of absorbance at 324 nm at 25°C for up to 2 hours. If depletion of PDA $\geq 30\%$, the test substance is classified as a sensitizer. If depletion of PDA $< 30\%$, confirmation tests, analogous to those with NBT, must be performed. For test substances that interfere with the absorbance of PDA, the loss of PDA is assessed by measuring the decrease in fluorescence at excitation wavelength = 324 nm and emission wavelength = 398 nm at 25°C for up to 2 hours.

Because the EASA does not use animals, it has the potential to greatly reduce animal use for ACD hazard testing. The EASA may serve as a screening assay in which positive results may potentially be acceptable without further testing. Results of the EASA could provide information that would be useful for integrated testing and decision strategies for ACD hazard.

Criteria 3: The extent of expected use and application of the proposed test methods and their impact on human health

The EASA would be used as an *in chemico* alternative to the LLNA to test substances for human ACD hazard. The EASA may also provide information on the ACD potency of

test substances. Appropriate ACD hazard labeling is important because the prognosis for ACD is poor (Hogan et al. 1990). More than 13 million workers in the US are potentially exposed to chemicals that can be absorbed through the skin, causing a variety of occupational diseases and disorders, including occupational skin diseases and systemic toxicity (NIOSH 2010). Occupational skin diseases, including ACD, are the most common types of occupational diseases, with estimated annual costs exceeding \$1 billion (NIOSH 2010). Consumers and workers are best protected from ACD by minimizing exposure to skin allergens.

Criteria 4: The potential for the proposed test methods to provide improved prediction of adverse health effects, compared to current accepted test methods

The EASA may be useful for detecting electrophilic skin allergens. Preliminary data show good accuracy, sensitivity, and specificity. It can provide essential protein reactivity information to improve integrated testing and decision strategies that reduce the use of animals. Applications include ACD hazard identification and, potentially, information that could be applied to human health risk assessment for exposure to skin allergens.

Criteria 5: The extent to which the proposed test methods provide advantages (e.g., reduced cost and time to perform) compared to current methods

The EASA does not require specialized training or the expertise that would be required to operate complex analytical systems such as high performance liquid chromatography (HPLC) or mass spectrometry (MS). It relies on a simple mix-and-read format. Solutions containing the substance and the protein surrogate, NBT or PDA, are dispensed into glass cuvettes and the decrease in absorbance or fluorescence is measured over 2 hours. Thus, the results are rapid.

The costs of the EASA are low. It does not require costly analytical equipment such as HPLC or MS. The necessary equipment includes a spectrophotometer and a fluorometer, both with temperature control. In addition, only millimolar solutions of test substance and protein surrogate are required. No animals and no radioactive reagents are required. The EASA may be amenable to increasing the throughput, now at the level of a research laboratory, through automation of liquid dispensing.

NICEATM Evaluation and Recommendations

Based on its preliminary evaluation, NICEATM concludes that this nomination should have a high priority for further evaluation of the usefulness and limitations of the EASA for ACD hazard classification. This evaluation will first require optimization and standardization of the test method protocol. An interlaboratory validation study will then be required to characterize the reliability and relevance of the test method.

Draft ICCVAM Prioritization and Draft Recommended Activities

The ICCVAM Interagency Immunotoxicity Working Group (IWG) has reviewed the nomination. Based on the information provided by the test method developer and consideration of the ICCVAM prioritization criteria, the ICCVAM IWG concludes that the nomination is of sufficient interest and applicability to warrant validation studies to characterize its usefulness and limitations for predicting ACD potential of chemicals and products. Accordingly, the ICCVAM IWG agrees with the NICEATM evaluation and concludes that this nomination should have a high priority for the proposed studies. After protocol optimization and standardization, an interlaboratory validation study should be designed to assess intra- and interlaboratory reproducibility and accuracy for the classification of ACD hazard. The ICCVAM IWG and NICEATM will contribute to the proposed studies by reviewing and commenting on (1) the optimization and standardization of the test method protocol, (2) the validation study design, and (3) the selection of reference chemicals for the validation study.

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