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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Key–Event-Based Test Guideline For In Chemico Skin Sensitisation Assays Addressing The Adverse Outcome Pathway Key Event On Covalent Binding To Proteins

GENERAL INTRODUCTION

Covalent binding to proteins Key Event based Test Guideline.

1.   A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). There is general agreement on the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised as an Adverse Outcome Pathway (AOP) (2) starting with a molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with amino-acid residues (i.e. cysteine or lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key event), is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signaling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event is T-cell proliferation.

2.   The assessment of skin sensitisation has typically involved the use of laboratory animals. The classical methods that use guinea-pigs, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman and the Buehler Test (OECD TG 406) (11) assess both the induction and elicitation phases of skin sensitisation. The murine tests, such as the LLNA (OECD TG 429) (12) and its three non-radioactive modifications — LLNA:DA (OECD TG 442A) (13), LLNA:BrdU-ELISA, and BrdU-FCM (OECD TG 442B) (14) — all assess the induction response exclusively and have gained acceptance, since they provide an advantage over the guinea pig tests in terms of animal welfare together with an objective measurement of the induction phase of skin sensitisation.

3.   Mechanistically-based in chemico and in vitro test methods addressing the first three key events of the skin sensitisation AOP have been adopted for contributing to the evaluation of the skin sensitisation hazard potential of chemicals: the present Test Guideline assesses covalent binding to proteins, addressing the first key event; the OECD TG 442D assesses keratinocyte activation (15), the second key event and the OECD TG 442E addresses the activation of dendritic cells (16), the third key event of the skin sensitisation AOP. Finally, the fourth key event representing T-cell proliferation is indirectly assessed in the murine Local Lymph Node Assay (LLNA) (12).

Background and principles of the test methods included in the Key Event based Test Guideline

4.   This Test Guideline (TG) describes in chemico assays that address mechanisms described under the first key event of the AOP for skin sensitisation, namely covalent binding to proteins (2). The Test Guideline comprises test methods to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS (1). The test methods currently described in this Test Guideline are:

•   The Direct Peptide Reactivity Assay (DPRA) (Appendix I), and

•   The Amino acid Derivative Reactivity Assay (ADRA) (Appendix II).

5.   These two test methods are based on in chemico covalent binding to proteins and are considered to be scientifically valid. The DPRA has been evaluated in a European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)-lead validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC) (3) (4) (5). The ADRA underwent a validation study coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM) (6) (7) (8) (9) followed by an independent peer-review (10).

6.   The test methods included in this Test Guideline might differ with regard to the procedures used to generate the data but can each be used to address countries' requirements for test results on protein reactivity, while benefiting from the Mutual Acceptance of Data.

7.   The correlation of protein reactivity with skin sensitisation potential is well established (17) (18) (19). Nevertheless, since protein reactivity represents only one key event of the skin sensitisation AOP (2) (20), information generated with test methods developed to address this specific key event may not be sufficient as stand-alone methods to conclude on the presence or absence of skin sensitisation potential of chemicals. Therefore data generated with the test methods described in this Test Guideline are proposed to support the discrimination between skin sensitisers (i.e. UN GHS Category 1) and non-sensitisers when used within Integrated Approaches to Testing and Assessment (IATA), together with other relevant complementary information from in vitro assays addressing other key events of the skin sensitisation AOP as well as non-testing methods, including in silico modeling and read-across from chemical analogues (20). Examples on the use of data generated with these methods within Defined Approaches (DAs) i.e. approaches standardised both in relation to the set of information sources used and in the procedure applied to derive predictions—have been published (20) and can be employed as useful elements within IATA.

8.   The test methods described in this Test Guideline do not allow either sub-categorisation of skin sensitisers into subcategories 1A and 1B (21), as defined by UN GHS (1) for authorities implementing these two optional subcategories, or potency prediction for safety assessment decisions. However, depending on the regulatory framework, positive results generated with these methods may be used on their own to classify a chemical into UN GHS Category 1.

9.   Definitions are provided in the Annex. Performance Standards for the assessment of proposed similar or modified in vitro skin sensitisation DPRA and ADRA test methods have been developed (22).

Literature for introduction

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ANNEX - DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with concordance to mean the proportion of correct outcomes of a test method (1).

(Formula shown below.)

ADRA: Amino acid Derivative Reactivity Assay

AOP (Adverse Outcome Pathway): sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an in vivo outcome of interest (2).

Calculation

Calculating depletion of either NAC or NAL

Depletion is calculated as follows:

Percent depletion of either NAC or NAL = {1- (NAC or NAL peak area in replicate injection ÷ mean NAC or NAL peak area in reference control C)} × 100

Calculating predictive capacity

There are several terms that are commonly used along with the description of sensitivity, specificity and accuracy. They are true positive (TP), true negative (TN), false negative (FN), and false positive (FP).

Sensitivity, specificity and accuracy are described in terms of TP, TN, FN, and FP.

Sensitivity: Number of true positives ÷ Number of all positive chemicals, TP ÷ (TP + FN)

Specificity: Number of true negatives ÷ Number of all negative chemicals, TN ÷ (TN + FP)

Accuracy: Number of correct predictions ÷ Number of all predictions, (TN + TP) ÷ (TN+TP+FN+FP)

Calibration curve: The relationship between the experimental response value and the analytical concentration (also called standard curve) of a known substance.

Coefficient of variation: a measure of variability that is calculated for a group of replicate data by dividing the standard deviation by the mean. It can be multiplied by 100 for expression as a percentage.

Defined Approach (DA): a DA consists of a fixed data interpretation procedure (e.g. statistical, mathematical models) applied to data (e.g. in silico predictions, in chemico, in vitro data) generated with a defined set of information sources to derive a prediction.

DPRA: Direct Peptide Reactivity Assay

EDTA: Ethylenediaminetetraacetic acid

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

IATA (Integrated Approach to Testing and Assessment): A structured approach used for hazard identification (potential), hazard characterisation (potency), and/or safety assessment (potential/potency and exposure) of a chemical or group of chemicals, which strategically integrates and weights all relevant data to inform regulatory decision regarding potential hazards, risks, and the need for further targeted and therefore minimal testing.

JaCVAM: Japanese Center for the Validation of Alternative Methods

LLNA: murine Local Lymph Node Assay issued as OECD TG 429 in 2010

Molecular Initiating Event: Chemical-induced perturbation of a biological system at the molecular level identified to be the starting event in the adverse outcome pathway.

Mixture: A solid or liquid comprising two or more substances which do not react chemically. (3)

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent comprises at least 80% (w/w) of the whole.

Multi-constituent substance: A substance, defined by its quantitative composition, in which two or more main constituents are present in concentrations $\geq 10\%$ (w/w) and $< 80\%$ (w/w). Multi-constituent substances are the result of a manufacturing process. The difference between a mixture and a multi-constituent substance is that a mixture comprises two or more substances which do not react chemically, whereas a multi-constituent substance comprises two or more substances that do react chemically.

NAC: N-(2-(1-naphthyl)acetyl)-L-cysteine (4) (5) (6)

NAL: α-N-(2-(1-naphthyl)acetyl)-L-lysine (4) (5) (6)

Positive control: A replicate containing all components of a test system and treated with a substance known to induce a positive response. To ensure that variability in the positive control response across time can be assessed, the magnitude of the positive response should not be excessive.

Pre-haptens: chemicals which become sensitisers through abiotic transformation

Pro-haptens: chemicals requiring enzymatic activation to exert skin sensitisation potential

Reference control: An untreated sample containing all components of a test system, including the solvent or vehicle that is processed with the test chemical treated and other control samples to establish the baseline response for the samples treated with the test chemical dissolved in the same solvent or vehicle. When tested with a concurrent negative control, this sample also demonstrates whether the solvent or vehicle interacts with the test system.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method. (1)

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability. (1)

Reproducibility: The concordance of results obtained from testing the same substance using the same test protocol (see reliability). (1)

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method. (1) (Formula shown below.)

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method. (1) (Formula shown below.)

Substance: Chemical elements and their compounds in the natural state or resulting from a manufacturing process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process, but excluding solvents that may be separated without affecting the stability of the substance or changing its composition (3).

System suitability: Determination of instrument performance (e.g. sensitivity) by analysis of a reference standard prior to running the analytical batch (7).

Test chemical: The term test chemical is used to refer to the substance being tested.

TFA: Trifluoroacetic acid

United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS): A system proposing the classification of chemicals (substances and mixtures) according to standardised types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (3).

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

Valid test method: A test method considered to have sufficient relevance and reliability for a specific purpose and which is based on scientifically sound principles. A test method is never valid in an absolute sense, but only in relation to a defined purpose (1).

Literature for definitions

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APPENDIX I

In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1.   The DPRA is proposed to address the molecular initiating event of the skin sensitisation AOP, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides containing either lysine or cysteine (1). Cysteine and lysine percent peptide depletion values are then used to categorise a substance in one of four classes of reactivity for supporting the discrimination between skin sensitisers and non-sensitisers (2).

2.   The DPRA test method proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. The level of reproducibility in predictions that can be expected from the test method is in the order of 85% within laboratories and 80% between laboratories (3). Results generated in the validation study (4) and published studies (5) overall indicate that the accuracy of the DPRA in discriminating sensitisers (i.e. UN GHS Cat. 1) from non-sensitisers is 80% (N=157) with a sensitivity of 80% (88/109) and specificity of 77% (37/48) when compared to LLNA results. The DPRA is more likely to under predict chemicals showing a low to moderate skin sensitisation potency (i.e. UN GHS subcategory 1B) than chemicals showing a high skin sensitisation potency (i.e. UN GHS subcategory 1A) (4) (5). However, the accuracy values given here for the DPRA as a stand-alone test method are only indicative since the test method should be considered in combination with other sources of information in the context of an IATA or a DA and in accordance with the provisions of paragraphs 7 and 8 in the General introduction. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in the species of interest, i.e. humans. On the basis of the overall data available, the DPRA was shown to be applicable to test chemicals covering a variety of organic functional groups, reaction mechanisms, skin sensitisation potency (as determined in *in vivo* studies) and physico-chemical properties (1) (2) (3) (5). Taken together, this information indicates the usefulness of the DPRA to contribute to the identification of skin sensitisation hazard.

3.   The term "test chemical" is used in this Test Guideline to refer to what is being tested[1] and is not related to the applicability of the DPRA to the testing of substances and/or mixtures. This test method is not applicable for the testing of metal compounds since they are known to react with proteins with mechanisms other than covalent binding. A test chemical should be soluble in an appropriate solvent at a final concentration of 100 mM (see paragraph 10). However, test chemicals that are not soluble at this concentration may still be tested at lower soluble concentrations. In such a case, a positive result could still be used to support the identification of the test chemical as a skin sensitiser but no firm

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[1] In June 2013, the Joint Meeting agreed that where possible, a more consistent use of the term "test chemical" describing what is being tested should now be applied in new and updated Test Guidelines.

conclusion on the lack of reactivity should be drawn from a negative result. Limited information is currently available on the applicability of the DPRA to mixtures of known composition (4) (5). The DPRA is nevertheless considered to be technically applicable to the testing of multi-constituent substances and mixtures of known composition (see paragraph 4 and 10). When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Appendix of the Test Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products or biological materials (i.e. UVCB substances) due to the defined molar ratio of test chemical and peptide. For this purpose a new prediction model based on a gravimetric approach will need to be developed. In cases where evidence can be demonstrated on the non-applicability of the test method to other specific categories of chemicals, the test method should not be used for those specific categories of chemicals.

4.   The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) are reported to be in most cases correctly detected by the test method (4) (9) (10). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA or a DA. Test chemicals that do not covalently bind to the peptide but promote its oxidation (i.e. cysteine dimerisation) could lead to a potential over estimation of peptide depletion, resulting in possible false positive predictions and/or assignment to a higher reactivity class (see paragraphs 21 and 22).

5.   As described, the DPRA assay supports the discrimination between skin sensitisers and non-sensitisers. However, it may also potentially contribute to the assessment of sensitising potency (6) (11) when used in integrated approaches such as IATA or DA (12). However further work, preferably based on human data, is required to determine how DPRA results may possibly inform potency assessment.

PRINCIPLE OF THE TEST

6.   The DPRA is an *in chemico* method which quantifies the remaining concentration of cysteine- or lysine-containing peptide following 24 hours incubation with the test chemical at 22.5-30°C. The synthetic peptides contain phenylalanine to aid in the detection. Relative peptide concentration is measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. Cysteine- and lysine peptide percent depletion values are then calculated and used in a prediction model (see paragraph 21) which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitisers and non-sensitisers.

7.   Prior to routine use of the method described in this Appenix, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 1.

PROCEDURE

8.　　This test method is based on the DPRA DB-ALM protocol nº 154 (7) which represents the protocol used for the EURL ECVAM-coordinated validation study. It is recommended that this protocol is used when implementing and using the method in the laboratory. The following is a description of the main components and procedures for the DPRA. If an alternative HPLC set-up is used, its equivalence to the validated set-up described in the DB-ALM protocol should be demonstrated (e.g. by testing the proficiency substances in Annex 1).

Preparation of the cysteine or lysine-containing peptides

9.　　Stock solutions of cysteine (Ac-RFAA**C**AA-COOH) and lysine (Ac-RFAA**K**AA-COOH) containing synthetic peptides of purity higher than 85% and preferably > 90%, should be freshly prepared just before their incubation with the test chemical. The final concentration of the cysteine peptide should be 0.667 mM in pH 7.5 phosphate buffer whereas the final concentration of the lysine peptide should be 0.667 mM in pH 10.2 ammonium acetate buffer. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to 26 analysis samples (which include the test chemical, the positive control and the appropriate number of solvent controls based on the number of individual solvents used in the test, each tested in triplicate), can be accommodated in a single HPLC run. All of the replicates analysed in the same run should use the identical cysteine and lysine peptide stock solutions. It is recommended to prove individual peptide batches for proper solubility prior to their use.

Preparation of the test chemical

10.　　Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay following the solubilisation procedure described in the DPRA DB-ALM protocol (7). An appropriate solvent will dissolve the test chemical completely. Since in the DPRA the test chemical is incubated in large excess with either the cysteine or the lysine peptides, visual inspection of the forming of a clear solution is considered sufficient to ascertain that the test chemical (and all of its components in the case of testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are, acetonitrile, water, 1:1 mixture water:acetonitrile, isopropanol, acetone or 1:1 mixture acetone:acetonitrile. Other solvents can be used as long as they do not have an impact on the stability of the peptide as monitored with reference controls C (i.e. samples constituted by the peptide alone dissolved in the appropriate solvent; see Annex 2). If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to peptide dimerisation and as a result, it may be more difficult to meet the acceptance criteria. If DMSO is chosen, attempts should be made to first solubilise the test chemical in 300 μL of DMSO and dilute the resulting solution with 2700 μL of acetonitrile. If the test chemical is not soluble in this mixture, attempts should be made to solubilise the same amount of test chemicals in 1500 μL of DMSO and dilute the resulting solution with 1500 μL of acetonitrile. The test chemical should be pre-weighed into glass vials and dissolved immediately before testing in an appropriate solvent to prepare a 100 mM solution. For mixtures and multi-constituent substances of known composition, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight should be determined by considering the individual molecular weights of each component

in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight should then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution. For polymers for which a predominant molecular weight cannot be determined, the molecular weight of the monomer (or the apparent molecular weight of the various monomers constituting the polymer) may be considered to prepare a 100 mM solution. However, when testing mixtures, multi-constituent substances or polymers of known composition, it should be considered to also test the neat chemical. For liquids, the neat chemical should be tested as such without any prior dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. For solids, the test chemical should be dissolved to its maximum soluble concentration in the same solvent used to prepare the apparent 100 mM solution. It should then be tested as such without any further dilution by incubating it at 1:10 and 1:50 ratio with the cysteine and lysine peptides, respectively. Concordant results (reactive or non-reactive) between the apparent 100 mM solution and the neat chemical should allow for a firm conclusion on the result.

Preparation of the positive control, reference controls and coelution controls

11.   Cinnamic aldehyde (CAS 104-55-2; ≥95% food-grade purity) should be used as positive control (PC) at a concentration of 100 mM in acetonitrile. Other suitable positive controls providing mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition reference controls (i.e. samples containing only the peptide dissolved in the appropriate solvent) should also be included in the HPLC run sequence and these are used to verify the HPLC system suitability prior to the analysis (reference controls A), the stability of the reference controls over time (reference control B) and to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion (reference control C) (see Annex 2). The appropriate reference control for each substance is used to calculate the percent peptide depletion for that substance (see paragraph 18). In addition, a co-elution control constituted by the test chemical alone for each of the test chemicals analysed should be included in the run sequence to detect possible co-elution of the test chemical with either the lysine or the cysteine peptide.

Incubation of the test chemical with the cysteine and lysine peptide solutions

12.   Cysteine and lysine peptide solutions should be incubated in glass autosampler vials with the test chemical at 1:10 and 1:50 ratio respectively. If a precipitate is observed immediately upon addition of the test chemical solution to the peptide solution, due to low aqueous solubility of the test chemical, one cannot be sure how much test chemical remained in the solution to react with the peptide. Therefore, in such a case, a positive result could still be used, but a negative result is uncertain and should be interpreted with due care (see also provisions in paragraph 10 for the testing of chemicals not soluble up to a concentration of 100 mM). The reaction solution should be left in the dark at 22.5-30°C for 24±2 hours before running the HPLC analysis. Each test chemical should be analysed in triplicate for both peptides. Samples have to be visually inspected prior to HPLC analysis. If a precipitate or phase separation is observed, samples may be centrifuged at low speed (100-400xg) to force precipitate to the bottom of the vial as a precaution since large amounts of precipitate may clog the HPLC tubing or columns. If a precipitation or phase separation is observed after the incubation period, peptide depletion may be underestimated

and a conclusion on the lack of reactivity cannot be drawn with sufficient confidence in case of a negative result.

Preparation of the HPLC standard calibration curve

13.　　A standard calibration curve should be generated for both the cysteine and the lysine peptides. Peptide standards should be prepared in a solution of 20% or 25% acetonitrile:buffer using phosphate buffer (pH 7.5) for the cysteine peptide and ammonium acetate buffer (pH 10.2) for the lysine peptide. Using serial dilution standards of the peptide stock solution (0.667 mM), 6 calibration solutions should be prepared to cover the range from 0.534 to 0.0167 mM. A blank of the dilution buffer should also be included in the standard calibration curve. Suitable calibration curves should have an r^2>0.99.

HPLC preparation and analysis

14.　　The suitability of the HPLC system should be verified before conducting the analysis. Peptide depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 220 nm signal). The appropriate column is installed in the HPLC system. The HPLC set-up described in the validated protocol uses a Zorbax SB-C-18 2.1 mm x 100 mm x 3.5 micron as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated at 30°C with 50% phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for at least 2 hours before running. The HPLC analysis should be performed using a flow rate of 0.35 mL/min and a linear gradient from 10% to 25% acetonitrile over 10 minutes, followed by a rapid increase to 90% acetonitrile to remove other materials. Equal volumes of each standard, sample and control should be injected. The column should be re-equilibrated under initial conditions for 7 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the cysteine and lysine peptides, including the injection volume, which may vary according to the system used (typically in the range from 3-10 μL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated (e.g. by testing the proficiency substances in Annex 1). Absorbance is monitored at 220 nm. If a photodiode array detector is used, absorbance at 258 nm should also be recorded. It should be noted that some supplies of acetonitrile could have a negative impact on peptide stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 220 peak area and the 258 peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90%<mean[2] area ratio of control samples<100% would give a good indication that co-elution has not occurred.

15.　　There may be test chemicals which could promote the oxidation of the cysteine peptide. The peak of the dimerised cysteine peptide may be visually monitored. If dimerisation appears to have occurred, this should be noted as percent peptide depletion may be over-estimated leading to false positive predictions and/or assignment to a higher reactivity class (see paragraphs 21 and 22).

16.　　The HPLC analysis should be timed to assure that the injection of the first sample starts 22 to 26 hours after the test chemical was mixed with the peptide solution. The HPLC run sequence should be set up in order to keep the HPLC analysis time less than 30 hours. For the HPLC set up used in the validation study and described in this test method, up to

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[2] For mean it is meant arithmetic mean throughout the document.

26 analysis samples can be accommodated in a single HPLC run (see also paragraph 9). An example of HPLC analysis sequence is provided in Annex 2.

DATA AND REPORTING

Data evaluation

17.   The concentration of cysteine or lysine peptide is photometrically determined at 220 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of peptide using the linear calibration curve derived from the standards.

18.   The percent peptide depletion is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant reference controls C (see Annex 2) according to the formula described below.

$$\text{Percent peptide depletion} = \left[1 - \left(\frac{\text{Peptide peak area in replicate injection}}{\text{Mean peptide peak area in reference controls } C} \right) \right] \times 100$$

Acceptance criteria

19.   The following criteria should be met for a run to be considered valid:

a) the standard calibration curve should have an $r^2 > 0.99$,

b) the mean percent peptide depletion value of the three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69.0% for the lysine peptide (for other positive controls a reference range needs to be established) and the maximum standard deviation (SD) for the positive control replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion and

c) the mean peptide concentration of reference controls A should be 0.50±0.05 mM and the coefficient of variation (CV) of peptide peak areas for the nine reference controls B and C in acetonitrile should be <15.0%.

If one or more of these criteria is not met the run should be repeated.

20.   The following criteria should be met for a test chemical's results to be considered valid:

a) the maximum standard deviation for the test chemical replicates should be <14.9% for the percent cysteine depletion and <11.6% for the percent lysine depletion,

b) the mean peptide concentration of the three reference controls C in the appropriate solvent should be 0.50±0.05 mM.

If these criteria are not met the data should be rejected and the run should be repeated for that specific test chemical.

Prediction model

21.   The mean percent cysteine and percent lysine depletion value is calculated for each test chemical. Negative depletion is considered as "0" when calculating the mean. By using the cysteine 1:10/lysine 1:50 prediction model shown in Table 1, the threshold of 6.38% average peptide depletion should be used to support the discrimination between skin sensitisers and non-sensitisers in the framework of an IATA or DA. Application of the prediction model for assigning a test chemical to a reactivity class (i.e. low, moderate and high reactivity) may perhaps prove useful to inform potency assessment within the framework of an IATA or DA.

Table 1: Cysteine 1:10/lysine 1:50 prediction model[1]

Mean of cysteine and lysine % depletion	Reactivity Class	DPRA Prediction[2]
0% ≤ mean % depletion ≤ 6.38%	No or minimal reactivity	Negative
6.38% < mean % depletion ≤ 22.62%	Low reactivity	Positive
22.62% < mean % depletion ≤ 42.47%	Moderate reactivity	
42.47% < mean % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

22.   There might be cases where the test chemical (the substance or one or several of the components of a multi-constituent substance or a mixture) absorbs significantly at 220 nm and has the same retention time of the peptide (co-elution). Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and the peptide. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated (e.g. by testing the proficiency substances in Annex 1). When co-elution occurs the peak of the peptide cannot be integrated and the calculation of the percent peptide depletion is not possible. If co-elution of such test chemicals occurs with both the cysteine and the lysine peptides then the analysis should be reported as "inconclusive". In cases where co-elution occurs only with the lysine peptide, then the cysteine 1:10 prediction model reported in Table 2 can be used.

Table 2: Cysteine 1:10 prediction model[1]

Cysteine (Cys) % depletion	Reactivity class	DPRA prediction[2]
0% ≤ Cys % depletion ≤ 13.89%	No or minimal reactivity	Negative
13.89% < Cys % depletion ≤ 23.09%	Low reactivity	Positive
23.09% < Cys % depletion ≤ 98.24%	Moderate reactivity	
98.24% < Cys % depletion ≤ 100%	High reactivity	

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.
[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

23.   There might be other cases where the overlap in retention time between the test chemical and either of the peptides is incomplete. In such cases percent peptide depletion values can be estimated and used in the cysteine 1:10/lysine 1:50 prediction model, however assignment of the test chemical to a reactivity class cannot be made with accuracy.

24.   A single HPLC analysis for both the cysteine and the lysine peptide should be sufficient for a test chemical when the result is unequivocal. However, in cases of results close to the threshold used to discriminate between positive and negative results (i.e. borderline results), additional testing may be necessary. If situations where the mean percent depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model or the cysteine percent depletion falls in the range of 9% to 17% for the cysteine 1:10 prediction model, a second run may be considered, as well as a third one in case of discordant results between the first two runs.

Test report

25.   The test report should include the following information

Test chemical

- Mono-constituent substance

  o   Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;

  o   Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;

  o   Purity, chemical identity of impurities as appropriate and practically feasible, etc;

  o   Treatment prior to testing, if applicable (e.g. warming, grinding);

  o   Concentration(s) tested;

  o   Storage conditions and stability to the extent available.

- Multi-constituent substance, UVCB and mixture:

  o   Characterisation as far as possible by e.g. chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available;

  o   Physical appearance, water solubility and additional relevant physicochemical properties, to the extent available;

  o   Molecular weight or apparent molecular weight in case of mixtures/polymers of known compositions or other information relevant for the conduct of the study;

  o   Treatment prior to testing, if applicable (e.g. warming, grinding);

  o   Concentration(s) tested;

  o   Storage conditions and stability to the extent available.

Controls

- Positive control

  - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;

  - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;

  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;

  - Treatment prior to testing, if applicable (e.g. warming, grinding);

  - Concentration(s) tested;

  - Storage conditions and stability to the extent available;

  - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

- Solvent/vehicle

  - Solvent/vehicle used and ratio of its constituents, if applicable;

  - Chemical identification(s), such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers;

  - Purity, chemical identity of impurities as appropriate and practically feasible, etc;

  - Physical appearance, molecular weight, and additional relevant physicochemical properties in the case other solvents/vehicles than those mentioned in the test method are used and to the extent available;

  - Storage conditions and stability to the extent available;

  - Justification for choice of solvent for each test chemical;

  - For acetonitrile, results of test of impact on peptide stability.

Preparation of peptides, positive control and test chemical

- Characterisation of peptide solutions (supplier, lot, exact weight of peptide, volume added for the stock solution);

- Characterisation of positive control solution (exact weight of positive control substance, volume added for the test solution);

- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test solution).

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler;

- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient.

System suitability

- Peptide peak area at 220 nm of each standard and reference control A replicate;

- Linear calibration curve graphically represented and the r^2 reported;

- Peptide concentration of each reference control A replicate;

- Mean peptide concentration (mM) of the three reference controls A, SD and CV;

- Peptide concentration of reference controls A and C.

Analysis sequence

- For reference controls:

  o Peptide peak area at 220 nm of each B and C replicate;

  o Mean peptide peak area at 220 nm of the nine reference controls B and C in acetonitrile, SD an CV (for stability of reference controls over analysis time);

  o For each solvent used, the mean peptide peak area at 220 nm of the three appropriate reference controls C (for the calculation of percent peptide depletion);

  o For each solvent used, the peptide concentration (mM) of the three appropriate reference controls C;

  o For each solvent used, the mean peptide concentration (mM) of the three appropriate reference controls C, SD and CV.

- For positive control:

  o Peptide peak area at 220 nm of each replicate;

  o Percent peptide depletion of each replicate;

  o Mean percent peptide depletion of the three replicates, SD and CV.

- For each test chemical:

  o Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;

  o Presence of co-elution;

  o Description of any other relevant observations, if applicable;

  o Peptide peak area at 220 nm of each replicate;

  o Percent peptide depletion of each replicate;

  o Mean of percent peptide depletion of the three replicate, SD and CV;

  o Mean of percent cysteine and percent lysine depletion values;

  o Prediction model used and DPRA prediction.

Proficiency testing

- If applicable, the procedure used to demonstrate proficiency of the laboratory in performing the test method (e.g. by testing of proficiency substances) or to demonstrate reproducible performance of the test method over time.

Discussion of the results

- Discussion of the results obtained with the DPRA test method;

- Discussion of the test method results in the context of an IATA if other relevant information is available.

Conclusion

LITERATURE FOR APPENDIX I

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APPENDIX I, ANNEX 1

PROFICIENCY SUBSTANCES

In Chemico **Skin Sensitisation: Direct Peptide Reactivity Assay**

Prior to routine use of the test method described in this test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected DPRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining cysteine and lysine depletion values that fall within the respective reference range for 8 out of the 10 proficiency substances for each peptide. These proficiency substances were selected to represent the range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality *in vitro* data generated with the DPRA are available, and that they were used in the EURL ECVAM-coordinated validation study to demonstrate successful implementation of the test method in the laboratories participating in the study.

Table 1: Recommended proficiency substances for demonstrating technical proficiency with the Direct Peptide Reactivity Assay

Proficiency substances	CASRN	Physical state	*In vivo* prediction[1]	DPRA prediction[2]	Range[3] of % cysteine peptide depletion	Range[3] of % lysine peptide depletion
2,4-Dinitrochlorobenzene	97-00-7	Solid	Sensitiser (extreme)	Positive	90-100	15-45
Oxazolone	15646-46-5	Solid	Sensitiser (extreme)	Positive	60-80	10-55
Formaldehyde	50-00-0	Liquid	Sensitiser (strong)	Positive	30-60	≤24
Benzylideneacetone	122-57-6	Solid	Sensitiser (moderate)	Positive	80-100	≤7
Farnesal	19317-11-4	Liquid	Sensitiser (weak)	Positive	15-55	≤25
2,3-Butanedione	431-03-8	Liquid	Sensitiser (weak)	Positive	60-100	10-45
1-Butanol	71-36-3	Liquid	Non-sensitiser	Negative	≤7	≤5.5
6-Methylcoumarin	92-48-8	Solid	Non-sensitiser	Negative	≤7	≤5.5
Lactic Acid	50-21-5	Liquid	Non-sensitiser	Negative	≤7	≤5.5
4-Methoxyacetophenone	100-06-1	Solid	Non-sensitiser	Negative	≤7	≤5.5

[1] The *in vivo* hazard and (potency) predictions are based on LLNA data (5). The *in vivo* potency is derived using the criteria proposed by ECETOC (8).

[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 4.

[3] Ranges determined on the basis of at least 10 depletion values generated by 6 independent laboratories.

EXAMPLES OF ANALYSIS SEQUENCE

Calibration standards and reference controls	STD1 STD2 STD3 STD4 STD5 STD6 Dilution buffer Reference control A, rep 1 Reference control A, rep 2 Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1 Reference control B, rep 2 Reference control B, rep 3
First set of replicates	Reference control C, rep 1 Cinnamic aldehyde, rep 1 Sample 1, rep 1 Sample 2, rep 1
Second set of replicates	Reference control C, rep 2 Cinnamic aldehyde, rep 2 Sample 1, rep 2 Sample 2, rep 2
Third set of replicates	Reference control C, rep 3 Cinnamic aldehyde, rep 3 Sample 1, rep 3 Sample 2, rep 3
Reference controls	Reference control B, rep 4 Reference control B, rep 5 Reference control B, rep 6

Three sets of reference controls (i.e. samples constituted only by the peptide dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: used to verify the suitability of the HPLC system.

Reference control B: included at the beginning and at the end of the analysis sequence to verify stability of reference controls over the analysis time.

Reference control C: included in the analysis sequence to verify that the solvent used to dissolve the test chemical does not impact the percent peptide depletion.

APPENDIX II

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1.   The ADRA is proposed to address the molecular initiating event of the skin sensitisation AOP—namely, protein reactivity—by quantifying the reactivity of test chemicals towards model synthetic amino acid derivatives containing either lysine or cysteine (1) (2) (3). Depletion values of cysteine and lysine derivatives are then used to support the discrimination between skin sensitisers and non-sensitisers (1) (2) (3).

2.   The ADRA proved to be transferable to laboratories experienced in high-performance liquid chromatography (HPLC) analysis. ADRA's WLR was 100% (10/10), 100% (7/7), 90% (9/10), and 100% (10/10) in four participating laboratories. BLR for 40 test chemicals calculated based the results from three participating laboratories was 91.9% (4). For the 40 chemicals tested in the validation study in four laboratories, the cumulative accuracy was 86.9% (139/160), sensitivity was 81.5% (88/108), and specificity was 98.1% (51/52) (4) (5). Results from the validation study (4) (5) as well as from other published studies (3) indicate that ADRA identified sensitisers and non-sensitisers with an accuracy of 79% (98/124) (124 compounds that fall within ADRA's applicability domain), a sensitivity of 74% (65/88), and a specificity of 92% (33/36) relative to LLNA results (6). In addition, the prediction of human skin sensitisation for 73 compounds that fall within ADRA's applicability domain has an accuracy of 86% (63/73), a sensitivity of 85% (44/52), and a specificity of 90% (19/21) (6). However, the accuracy values given here for ADRA as a stand-alone test method are for reference only, since it is recommended that the test method be used in combination with other sources of information in the context of an IATA and in accordance with the provisions of paragraphs 7 and 8 in the General Introduction. Furthermore when evaluating non-animal methods for skin sensitisation, it should be kept in mind that the LLNA test as well as other animal tests may not fully reflect the situation in the species of interest, which is humans. On the basis of the overall data available, ADRA's applicability domain was shown to include a variety of organic functional groups, reaction mechanisms, skin sensitisation potencies (as determined in *in vivo* studies), and physicochemical properties (1) (2) (3) (4). Following an independent peer review, the ADRA validation study was considered to demonstrate that this method should be acceptable as part of an integrated testing strategy for the predictive identification of skin sensitisation hazard (7).

3.   The term "test chemical" is used in this Test Guideline to refer to what is being tested and is not related to the applicability of the ADRA to the testing of substances and/or mixtures. This test method is not applicable to the testing of metal compounds, which are known to react with proteins via mechanisms other than covalent binding. The test method described in this Appendix of the Test Guideline is an *in chemico* method that does not encompass a metabolic system. Chemicals that require enzymatic bioactivation to exert their skin sensitisation potential (i.e. pro-haptens) cannot be detected by the test method. Chemicals that become sensitisers after abiotic transformation (i.e. pre-haptens) are

reported to be in some cases correctly detected by the test method (1) (2) (3) (4). In the light of the above, negative results obtained with the test method should be interpreted in the context of the stated limitations and in the connection with other information sources within the framework of an IATA. Test chemicals that promote the oxidation of the *N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) reagent (i.e. cysteine dimerisation) could lead to a potential over-estimation of NAC depletion, resulting in possible false positive predictions (see paragraphs 27 and 28); it may be possible to detect and quantify any NAC dimer formed by HPLC, thus confirming or ruling out that the NAC reagent has been depleted via oxidative dimerisation as opposed to reaction and covalent bonding to the test item substance(s).

4. The ADRA test method allows testing of poorly soluble chemicals. To be tested, a test chemical should be soluble in an appropriate solvent at a final concentration of 1 mM (see paragraph 14). Test chemicals that are not soluble at this concentration may still be tested at lower concentrations. In such cases, a positive result could still be used to support identification of the test chemical as a skin sensitiser but no firm conclusion on the lack of reactivity should be drawn from a negative result.

5. In general, many organic compounds absorb UV in the range of 220 nm. In the case of co-elution of the nucleophilic reagent and the test chemical, this might result in false negative prediction. This may happen with the DPRA which specifies that quantification of the peptide-based nucleophilic reagents has to be performed at 220 nm. In contrast to this, the nucleophilic reagents used in ADRA are quantified at 281 nm. The substances that absorb UV in this range of the spectrum are generally limited to those having conjugated double bonds, which significantly lowers the potential for co-elution (8).

6. The current prediction model cannot be used for complex mixtures of unknown composition or for substances of unknown or variable composition, complex reaction products, or biological materials (UVCB substances) due to the need for defined molar ratio of test chemical and nucleophilic reagents. Limited information is currently available on the applicability of the ADRA to mixtures (9) (10). A new protocol has to be developed for multi-constituent substances and mixtures to be used with test methods like ADRA, which utilise HPLC analysis to quantify the depletion of nucleophilic reagents (9) (10). Thus, although it is impossible to define fixed methods in this guideline, which can evaluate multi-constituent substances and mixtures, paragraph 15 describes an evaluation method that is considered to be applicable at the present time for multi-constituent substances or mixtures of known composition (9). Such substances were however not tested during the validation studies. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

7. ADRA can be used to support the discrimination between skin sensitisers and non-sensitisers. Further work, preferably based on human data, is necessary to determine whether ADRA results can contribute to potency assessment when considered in combination with other information sources.

PRINCIPLE OF THE TEST

8.   ADRA is an *in chemico* test method that quantifies residual concentrations of the cysteine derivative *N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (CAS. 32668-00-1), which is known as NAC, and the lysine derivative α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine (CAS. 397841-92-8), known as NAL, following a 24±1 hour incubation at 25±1ºC in the presence of a test chemical. Both these derivatives include a naphthalene ring that is introduced to their *N*-terminal in order to facilitate UV detection. The relative concentrations of NAC and NAL are measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 281 nm. Percent depletion values are then calculated for both NAC and NAL and compared to a prediction model (see paragraph 26).

9.   Prior to routine use of the method described in this test method, laboratories should demonstrate technical proficiency, using the ten proficiency substances listed in Annex 1 of this Appendix.

PROCEDURE

10.   This test method is based on the protocol (11) used for the JaCVAM-coordinated ADRA validation study and is recommended for use when implementing ADRA at a laboratory. The main components and procedures for the ADRA are described below. Before using an alternative HPLC set-up, its equivalence to the validated set-up described in the protocol should be demonstrated, preferably by testing the proficiency substances in Annex 1 of this Appendix.

Quality of NAC and NAL

11.   The Nucleophilic Reagents can be obtained as an ADRA Kit for Skin Sensitisation Test, from FUJIFILM Wako (FFWK) Pure Chemical Corporation, Catalog No. 296-80901. Manufacturing NAC/NAL is patented in Japan only, by Fujifilm Corporation. Therefore, manufacturers in other countries can produce NAC/NAL without permission. In case other NAC/NAL are used, these should satisfy three quality criteria described below. Quality checks can be obviated and ADRA testing performed without delay by purchasing NAC and NAL that have been manufactured specifically to satisfy these quality criteria.

Quality required for NAC and NAL:

1) Purity: Both NAC and NAL are to be at least 98% pure.

2) Stability: Using NAC and NAL stock solution, prepare a reference control free of any test chemical and quantify the residual levels of NAC and NAL both immediately after preparation (0 hours) and after a 24 hour incubation. Residual levels of NAC and NAL are to be a minimum of 90% in either case (11). The residual level of NAC is calculated as a percentage of the sum of NAC and the residual level of NAC dimers.

3) Reactivity: NAC and NAL are to be evaluated for reactivity with the ten proficiency substances given in Annex 1 and should satisfy the requirement given therein.

Preparation of the NAC and NAL stock solution

12.   The solubility of individual NAC and NAL batches should be verified prior to use. NAC stock solution should be prepared to a concentration of 2 mM in 100 mM of pH 8.0 phosphate buffer, including 0.333 μM of EDTA, as well as NAL stock solution to a concentration of 2 mM in 100 mM of pH 10.2 phosphate buffer. These two stock solutions are then diluted in buffer to prepare 6.667 μM stock solutions. Both NAC and NAL stock solutions should be used as soon as possible after preparation (3). In the event that they are to be stored, these stock solutions may be frozen and stored for up to twelve months time at less than -75°C prior to use. The final concentration of the NAC solution is 5 μM in pH 8.0 phosphate buffer, and the final concentration of the NAL solution is 5 μM in pH 10.2 phosphate buffer.

Preparation of the test chemical solution

13.   Solubility of the test chemical in an appropriate solvent should be assessed before performing the assay in accordance with the solubilisation procedure described in the ADRA JaCVAM protocol (11). An appropriate solvent should dissolve the test chemical completely. Since the ADRA protocol stipulates that the test chemical be incubated in an excess volume of both NAC and NAL, visual inspection of the clear test chemical solution is considered sufficient to confirm that the test chemical (and all its constituents, if testing a multi-constituent substance or a mixture) is dissolved. Suitable solvents are distilled water, acetonitrile and acetone. If the test chemical is not soluble in any of the solvents mentioned above, DMSO can be used as a last resort and in minimal amounts. It is important to note that DMSO may lead to dimerisation of the nucleophilic reagent NAC (12) and as a result, it may be more difficult to meet the acceptance criteria. If DMSO is chosen, attempts should be made to solubilise the test chemical in a 1:20 mixture of DMSO and acetonitrile (5% DMSO in acetonitrile). When using a DMSO-acetonitrile solvent, the test chemical should be dissolved in DMSO, and then this solution should be diluted 20-fold with acetonitrile to prepare a 1 mM test chemical solution. In case the use of DMSO leads to increased dimerisation of the NAC reagent, this can be checked analytically as the NAC dimer can be detected by HPLC. The test chemical should be pre-weighed into a disposable polypropylene tube and dissolved immediately before testing in an appropriate solvent to prepare a 1 mM solution.

14.   Mono-constituent substances of unknown molecular weight may be tested in a test chemical solution at a concentration of 0.5 mg/mL rather than 1 mM (9). Polymers which are well characterised should also be tested at a concentration of 1 mM based on the mean number average molecular weight, in a manner analogous to the procedure for mono-constituent compounds.

15.   Mixtures and multi constituent substances, of known composition are to be tested as follows:

> 1) Liquids: Generally, tested as an undiluted mixture. In cases where low solubility of the test item prevents formation of reaction solution, i.e. undissolved material, clouding, and/or precipitation is observed, a positive result may still be used in the assessment, whereas a negative result is uncertain and should be interpreted with due care. Insofar as results could be false positives, however, predictions should be interpreted with due care.

> 2) Solids: The test chemical should be dissolved to maximum soluble concentration in the same solvent used to prepare the 1 mM test chemical solution. The test

chemical solution of the highest concentration possible is then tested as an undiluted mixture. In cases where low solubility of the test item prevents formation of reaction solution, i.e. undissolved material, clouding, and/or precipitation is observed, a positive result may still be used in the assessment, whereas a negative result is uncertain and should be interpreted with due care. Insofar as results could be false positives, however, predictions should be interpreted with due care.

Preparation of the positive control, reference controls and co-elution controls

16. Phenylacetaldehyde (CAS 122-78-1, ≥90% purity) should be used as positive control (PC) at a concentration of 1 mM in acetonitrile. Other suitable positive controls that provide mid-range depletion values may be used if historical data are available to derive comparable run acceptance criteria. In addition, reference controls comprising only NAC or only NAL dissolved in the appropriate solvent should also be included in the HPLC run sequence, so they can be used to verify the HPLC system suitability prior to analysis (Reference Control A), the stability of the reference controls over time (Reference Control B), and any effects of the solvent used on depletion of NAC or NAL (Reference Control C) (See Annex 2). The percent NAC and NAL depletion for a test chemical is calculated using an appropriate reference control for that test chemical (see paragraph 23). Also, a co-elution control comprising only the test chemical should be included in the run sequence to detect possible co-elution of the test chemical with either the NAC or NAL.

Incubation of the test chemical with the NAC and NAL solutions

17. Both the NAC and the NAL solutions should be incubated with the test chemical at 1:50 ratio in a 96-well microplate. The observation of precipitate immediately upon addition of the test chemical solution to the NAC and the NAL solutions is an indication of poor solubility, which means that there is no way to know exactly how much test chemical is contained in the solution. Thus, although positive results can be used with confidence, negative results are uncertain and should be interpreted with due care (see also paragraph 4 regarding the testing of chemicals not soluble at concentrations as high as 1 mM). The reaction solution should be incubated in the dark at 25±1ºC for 24±1 hours before performing HPLC analysis. After incubation, trifluoroacetic acid (TFA) (≥ 98%) should be added as a fixing solution to stop the reaction (3).

HPLC preparation and analysis

18. Each test chemical should be analysed in triplicate to determine percent depletion for both NAC and NAL. Although adding the fixing solution does stop the reaction, measurement of the reaction solution is to be performed as soon as possible and in any case within three days after adding the fixing solution. For example, when HPLC analysis of NAC and NAL are performed separately using two 96-well microplates, up to 34 samples may be analysed at one time, including the test chemical, the positive control, and the appropriate number of solvent controls based on the number of individual solvents used in the test, each in triplicate. All of the replicates analysed in a single run should use identical batches of NAC and NAL stock solution. Test chemical and control solutions are to be visually inspected prior to HPLC analysis and may be centrifuged at low speed (100–400 × g) to force any precipitate to the bottom of the vial as a precaution against large amounts of precipitate clogging the HPLC tubing or columns. Observation of precipitation or phase separation after the incubation period is an indication that NAC and NAL depletion could be misleading, and negative results in that case are uncertain and should be interpreted with

due care, as well as for any precipitate observed at the beginning of the incubation period (see above).

19.   A standard calibration curve should be generated for both NAC and NAL. Standard solutions of both NAC and NAL should be prepared in 20% acetonitrile in buffer and containing 0.5% trifluoroacetic acid. For NAC, a phosphate buffer at pH 8.0, and for NAL, a phosphate buffer at pH 10.2 should be used. Serial dilution of the NAC and NAL stock solutions (5.0 μM) will be used to prepare six calibration solutions in concentrations from 5.0 to 0.156 μM as well as a blank of the dilution buffer. Suitable calibration curves should have an $R^2 > 0.990$.

20.   The suitability of the HPLC system should be verified before conducting the analysis. Both NAC and NAL depletion is monitored by HPLC coupled with an UV detector (photodiode array detector or fixed wavelength absorbance detector with 281 nm signal). The appropriate column is installed in the HPLC system. The recommended HPLC set-up described in the validated protocol uses a column (Base particle: core-shell type silica gel, Particle size: 2.5~2.7 μm, colomn size: 3.0 × 150 mm) as preferred column. With this reversed-phase HPLC column, the entire system should be equilibrated for at least 30 minutes at 40ºC with 50% phase A (0.1% (v/v) trifluoroacetic acid in water), 50% phase B (0.1% (v/v) trifluoroacetic acid in acetonitrile) before use. Then, the column is conditioned by running the gradient at least twice before actual use. The HPLC analysis should be performed using a flow rate of 0.30 mL/min and a linear gradient from 30% to 55% acetonitrile for NAC and from 25% to 45% acetonitrile for NAL within 10 minutes, followed by a rapid increase to 100% acetonitrile to remove other materials. Equal volumes of the standard solutions, test chemical solutions, and control solutions should be injected. The column should be re-equilibrated under initial conditions for 6.5 minutes between injections. If a different reversed-phase HPLC column is used, the set-up parameters described above may need to be adjusted to guarantee an appropriate elution and integration of the NAC and NAL, including the injection volume, which may vary according to the system used (typically in the range from 10–20 μL). Importantly, if an alternative HPLC set-up is used, its equivalence to the validated set-up described above should be demonstrated, preferably by testing the proficiency substances in Annex 1. Absorbance is monitored at 281 nm. If a photodiode array detector is used, absorbance at 291 nm should also be recorded. It should be noted that some batches of acetonitrile could have a negative impact on NAC and NAL stability and this has to be assessed when a new batch of acetonitrile is used. The ratio of the 281 nm peak area and the 291 nm peak area can be used as an indicator of co-elution. For each sample a ratio in the range of 90% < mean area ratio of control samples < 100% would give a good indication that co-elution has not occurred. An example of HPLC analysis sequence is provided in Annex 2.

21.   There are some test chemicals that could potentially promote oxidation of NAC. The peak of the dimerised NAC may be monitored visually. Any apparent dimerisation should be noted, since overestimation of NAC depletion could result in false-positive predictions (See paragraphs 26 and 27).

DATA AND REPORTING

Data evaluation

22.   The concentration of both NAC and NAL is photometrically determined at 281 nm in each sample by measuring the peak area (area under the curve, AUC) of the appropriate peaks and by calculating the concentration of both NAC and NAL using the linear calibration curve derived from the standards.

23.   The percent depletion for both NAC and NAL is determined in each sample by measuring the peak area and dividing it by the mean peak area of the relevant Reference Controls C (See Annex 2) according to the formula described below.

$$\text{Percent NAC or NAL depletion} = \left[1 - \left[\frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in reference controls C}}\right]\right] \times 100$$

Acceptance criteria

24.   The following criteria should be met:

a) the standard calibration curve should have an $R^2 > 0.990$,

b) the mean percent NAC and NAL depletion value of the three replicates for the positive control phenylacetaldehyde should be between 6% and 30% for NAC and between 75% and 100% for NAL, while the maximum standard deviation (SD) for the positive control replicates should be < 10% for both NAC and NAL depletion, and

c) the mean NAC and NAL concentration of both Reference Controls A and Reference Control C should be 3.2–4.4 μM and the coefficient of variation (CV) of NAC and NAL peak areas for the nine Reference Controls B and C in acetonitrile should be < 10%.

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated.

25.   The following criteria should be satisfied for a test chemical's results to be accepted as valid:

a) the maximum standard deviation for the test chemical replicates should be < 10% for the percent depletion of both NAC and NAL,

b) the mean NAC and NAL concentration of the three Reference Controls C in the appropriate solvent should be 3.2–4.4 μM.

If one or more of these criteria is not satisfied, the data should be rejected and the run should be repeated.

Prediction model

26.   The mean percent depletion of NAC and NAL is calculated for each test chemical. Negative depletion is considered to be "0" when calculating the mean. By using the NAC/NAL prediction model shown in Table 1, the threshold of 4.9% mean percent depletion should be used to support the discrimination between skin sensitisers and non-sensitiser in the framework of an IATA or a DA.

Table 1: NAC/NAL prediction model[1]

Mean NAC and NAL percent depletion	ADRA prediction[2]
Less than 4.9%	Negative
4.9% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

27.   Co-elution occurs when the test chemical (the substance or one or several of the constituents of a multi-constituent substance or a mixture) absorbs significantly at 281 nm and has the same retention time as NAC or NAL. Co-elution may be resolved by slightly adjusting the HPLC set-up in order to further separate the elution time of the test chemical and NAC or NAL. If an alternative HPLC set-up is used to try to resolve co-elution, its equivalence to the validated set-up should be demonstrated, preferably by testing the proficiency substances in Annex 1. When co-elution occurs, it is not possible to integrate the peak of the NAC or NAL, thereby preventing calculation of the percent depletion of NAC or NAL. If co-elution of test chemicals occurs with both the NAC and NAL and separation of elution time is not feasible, then the analysis should be reported to be inconclusive. In cases where co-elution occurs only with NAL and separation of elution time is not feasible, the NAC-only prediction model (See Table 2) can be used to make a prediction.

Table 2: NAC-only prediction model[1]

Mean NAC percent depletion	ADRA prediction[2]
Less than 5.6%	Negative
5.6% or higher	Positive

[1] The numbers refer to statistically generated threshold values and are not related to the precision of the measurement.

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

28.   When a result is unequivocal, a single HPLC analysis for both NAC and NAL should be sufficient for a test chemical. Additional testing is sometimes necessary, however, when the results lie close to the threshold value used to discriminate between positive and negative results (borderline results). If the mean percent depletion falls between 3.0% and 10.0% when using the NAC/NAL prediction model or the NAC percent depletion falls between 4.0% and 11.0% when using the NAC-only prediction model, a second run is advisable, as is a third run in the event of discordant results between the first two runs.

Test report

29.   The test report should include the following information:

Test chemical

- Mono-constituent substance
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers
    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available
    - Purity, chemical identity of impurities as appropriate and practically feasible, etc.
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability to the extent available

- Multi-constituent substance, UVCB, and mixtures
    - Characterisation by chemical identity (see above), purity, quantitative occurrence and relevant physicochemical properties (see above) of the constituents, to the extent available
    - Physical appearance, water solubility, and additional relevant physicochemical properties, to the extent available
    - Molecular weight (or apparent molecular weight) for mixtures or polymers of known composition, or other information relevant to the study
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability, to the extent available.

Controls

- Positive control
    - Chemical identification, such as IUPAC or CAS name(s), CAS number(s), SMILES or InChI code, structural formula, and/or other identifiers;
    - Physical appearance, water solubility, molecular weight, and additional relevant physicochemical properties, to the extent available;
    - Purity, chemical identity of impurities, as appropriate or feasible
    - Treatment prior to testing, if applicable (warming, grinding)
    - Concentration(s) tested
    - Storage conditions and stability, to the extent available;
    - Reference to historical positive control results demonstrating suitable run acceptance criteria, if applicable.

- Solvent

    - Solvent used and ratio of its constituents, if applicable

    - Chemical identification(s), such as IUPAC or CAS name(s), CAS number(s), and/or other identifiers

    - Purity, chemical identity of impurities, as appropriate and feasible

    - Physical appearance, molecular weight, and additional relevant physicochemical properties when solvents other than those mentioned in the test method are used

    - Storage conditions and stability, to the extent available

    - Justification for choice of solvent for each test chemical

    - Impact on NAC and NAL stability when using acetonitrile

Preparation of NAC and NAL, positive control and test chemical solution

- Characterisation of NAC and NAL solutions (supplier, lot, exact weight of NAC and NAL, volume added for the stock solution)

- Characterisation of positive control solutions (exact weight of positive control reagent, volume added for the control solution)

- Characterisation of test chemical solutions (exact weight of test chemical, volume added for the test chemical solution)

HPLC instrument setting and analysis

- Type of HPLC instrument, HPLC and guard columns, detector, autosampler

- Parameters relevant for the HPLC analysis such as column temperature, injection volumes, flow rate and gradient

System suitability

- NAC and NAL peak area at 281 nm of each standard and reference control A replicate

- Linear calibration curve graphically represented and the R2 reported

- NAC and NAL concentration of each Reference Control A replicate

- Mean NAC and NAL concentration (μM) of the three reference controls A, SD and CV

- NAC and NAL concentration of Reference Controls A and C.

Analysis sequence

- For Reference Controls

    - NAC and NAL peak area at 281 nm of each replicate of Reference Controls B and C

    - Mean NAC and NAL peak area at 281 nm of the nine Reference Controls B and C in acetonitrile, SD and CV (for stability of reference controls over analysis time)

- o For each solvent used, the mean NAC and NAL peak area at 281 nm of the three appropriate Reference Controls C (for the calculation of percent NAC and NAL depletion)

- o For each solvent used, the NAC and NAL concentration (μM) of the three appropriate Reference Controls C

- o For each solvent used, the mean NAC and NAL concentration (μM) of the three appropriate Reference Controls C, SD and CV.

- For positive controls

  - o NAC and NAL peak area at 281 nm of each replicate

  - o Percent NAC and NAL depletion of each replicate

  - o Mean percent NAC and NAL depletion of the three replicates, SD and CV.

- For each test chemical

  - o Appearance of precipitate in the reaction mixture at the end of the incubation time, if observed. If precipitate was re-solubilised or centrifuged;

  - o Presence of co-elution

  - o Description of any other relevant observations, if applicable

  - o NAC and NAL peak area at 281 nm of each replicate

  - o Percent NAC and NAL depletion of each replicate

  - o Mean of percent NAC and NAL depletion of the three replicate, SD and CV

  - o Mean of percent NAC and percent NAL depletion values

  - o Prediction model used and ADRA prediction

Proficiency testing

- If applicable, the procedure used to demonstrate proficiency of the laboratory in performing the test method (testing of proficiency substances, etc.) or to demonstrate reproducible performance of the test method over time.

Discussion of the results

- Discussion of the results obtained with the ADRA test method

- Discussion of the test method results in the context of an IATA if other relevant information is available

Conclusion

Literature for Appendix II

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APPENDIX II, ANNEX 1

Proficiency Substances

In Chemico Skin Sensitisation: Amino acid Derivative Reactivity Assay (ADRA)

Prior to routine use of the test method, laboratories should demonstrate technical proficiency by correctly obtaining the expected ADRA prediction for the 10 proficiency substances recommended in Table 1 and by obtaining NAC and NAL depletion values that fall within the respective reference ranges for 8 out of the 10 proficiency substances. These proficiency substances were selected to represent the full range of responses for skin sensitisation hazards. Other selection criteria were that they are commercially available, that high quality *in vivo* reference data and high quality ADRA data are available, and that they were used during the JaCVAM-coordinated validation study to demonstrate successful implementation.

Table 1. Recommended chemicals for demonstrating technical proficiency with ADRA

No.	Test chemicals	CAS No.	Physical state	Molecular weight	*In vivo* Prediction[1]	ADRA prediction[2]	Range of % depletion	
							NAC[3]	NAL[3]
1	*p*-Benzoquinone	106-51-4	Solid	108.09	Sensitiser (extreme)	Positive	90-100	40-70
2	Chloramine T trihydrate	7080-50-4	Solid	281.69	Sensitiser (strong)	Positive	90-100	90-100
3	Trans-Cinnamaldehyde	14371-10-9	Liquid	132.16	Sensitiser (moderate)	Positive	40-100	≤20
4	Palmitoyl Chloride	112-67-4	Liquid	274.87	Sensitiser (moderate)	Positive	≤10	50-100
5	Imidazolidinyl urea	39236-46-9	Solid	388.29	Sensitiser (weak)	Positive	10-45	≤10
6	Farnesal	19317-11-4	Liquid	220.35	Sensitiser (weak)	Positive	20-40	≤15
7	Glycerol	56-81-5	Liquid	92.09	Non-sensitiser	Negative	≤7	≤7
8	Benzyl alcohol	100-51-6	Liquid	108.14	Non-sensitiser	Negative	≤7	≤7
9	Dimethyl isophthalate	1459-93-4	Solid	194.19	Non-sensitiser	Negative	≤7	≤7
10	Propyl paraben	94-13-3	Solid	180.20	Non-sensitiser	Negative	≤7	≤7

[1]The *in vivo* hazard (and potency) predictions are based on LLNA data. (13) (14) (15). The *in vivo* potency is derived using the criteria proposed by ECETOC (16).

[2] An ADRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 2 and 3.

[3] Ranges determined on the basis of at least 10 depletion values generated by 5 independent laboratories.

APPENDIX II, ANNEX 2

EXAMPLES OF ANALYSIS SEQUENCE

Each sample of HPLC analysis should be analysed in number order below. Refer to the table showing Examples of HPLC Sample Analysis Sequences for more practical sequences about HPLC analysis.

1. Start to analyse calibration standards and Reference Control A (N = 3).

2. The co-elution Control does not need to be analysed by turns if it is analysed after analysis of standard solution and Reference Control A.

3. Reference Control B should be analysed three times (total six times) before and after the analysis of sample, Reference Control C and Positive Control.

4. The Reference Control C, Positive Control and Test chemical solutions are analysed. (After the first set of replicates of each sample is analysed, the second set of replicates of each should be analysed).

Calibration standards and reference controls	STD1
	STD2
	STD3
	STD4
	STD5
	STD6
	Dilution buffer
	Reference control A, rep 1
	Reference control A, rep 2
	Reference control A, rep 3
Co-elution controls	Co-elution control 1 for test chemical 1 Co-elution control 2 for test chemical 2
Reference controls	Reference control B, rep 1
	Reference control B, rep 2
	Reference control B, rep 3
First set of replicates	Reference control C, rep 1
	Phenylacetaldehyde, rep 1
	Sample 1, rep 1
	Sample 2, rep 1
Second set of replicates	Reference control C, rep 2
	Phenylacetaldehyde, rep 2
	Sample 1, rep 2
	Sample 2, rep 2
Third set of replicates	Reference control C, rep 3
	Phenylacetaldehyde, rep 3
	Sample 1, rep 3
	Sample 2, rep 3
Reference controls	Reference control B, rep 4
	Reference control B, rep 5
	Reference control B, rep 6

Three sets of reference controls (NAC or NAL dissolved in the appropriate solvent) should be included in the analysis sequence:

Reference control A: Control for verifying validity of the HPLC system. Reference Control A is used to verify concentration of NAC and NAL from each calibration curve after addition of acetonitrile rather than test chemical.

Reference control B: Control for verifying stability of reaction solution under analysis. Reference Control B is used to verify variability (CV) of each three NAC/NAL peak areas in the solution after addition of acetonitrile rather than test chemical at the start of analysis and at the end of analysis.

Reference control C:

Control for calculating NAC/NAL depletion of each test chemical solution. To calculate depletion of NAC/NAL, measure three Reference Controls C after addition of solvent instead of test chemical. Prepare reference Control C for all solvents used to dissolve the test chemicals.