Regarding the references for reference chemicals of alternative methods

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ABSTRACT

The selection of reference and proficiency chemicals is an important component of method validation and proficiency evaluations. Reference chemicals are a set of test substances used by a method developer to evaluate the reliability and relevance of a new method, in comparison to reference data (usually to a validated reference method). Proficiency chemicals, as defined in OECD Guidance Document on Good In Vitro Method Practices, are defined post validation as a subset of the reference chemicals, or other chemicals with sufficient supporting data, that are used by naïve laboratories to demonstrate technical competence with a validated test method. Proficiency chemicals should cover different physical states, several chemical classes within the applicability domain of the method and yield the full range of responses (in the validated reference method and in vivo). They shall be commercially available (at non-prohibitive costs) and have high quality reference data. If reference and subsequent proficiency chemicals are chosen without sufficient evidence for their inclusion, both test method evaluation and demonstration of technical proficiency can be hampered. In this report we present cases in which the selection of reference chemicals led to problems in the reproduction of the reference results and demonstration of technical proficiency.

LLNA Performance Standards (OECD TG 429)

The Local Lymph Node Assay (LLNA) to determine sensitization is a mouse based assay traditionally measuring the incorporation of radiolabelled thymidine (OECD TG 429) into proliferating lymph nodes. PS have been tested to show that different endpoints of cell proliferation yield the same results (data not shown). Our evaluation, however, revealed that the variability in the results for LLNA according to OECD TG 429 was not always taken into account when the PS were chosen.

Substance[a]	PS LLNA Reference Data SI (concentrations tested [%])[c, d]	Concurrent LLNA SI (concentrations tested [%])[c]	Discussion
Chlorobenzene	1.1, 1.7, 1.6 (tested at 5, 10, 25)	1.9, 3.2, 5.3 (tested at 25, 50, 100)[e]	▪ PS reference data obtained from 1 single study ▪ higher concentration tested in concurrent LLNA ▪ insufficient human epidemiologic evidence for classification as sensitizer
Methyl salicylate	0.7-2.1, 0.7-2.7, 0.8-2.6, 0.5-1.9, 0.9-2.9 (tested at 1.0, 2.5, 5.0, 10, 20)	1.2, 1.8, 5.6 (tested at 10, 25, 50)[e]	▪ PS reference data obtained from 9 studies (6 tested up to 20% and 3 tested up to 5%) ▪ PS LLNA borderline negative ▪ higher concentration tested in concurrent LLNA ▪ insufficient human epidemiologic evidence for classification as sensitizer
Methyl methacrylate	1.4, 1.5, 1.5, 2.1, 3.6 (tested at 10, 30, 50, 75, 100)[f]	1.3, 1.3, 2.3 (tested at 25, 50, 100)[e] 0.7, 0.7, 2.2 (tested at 25, 50, 100)[g]	▪ PS reference data obtained from 1 single study ▪ PS LLNA borderline positive ▪ borderline / weak human sensitizer
Nickel chloride	1.0, 1.7, 2.2 (tested at 0.5, 1.0, 2.5) 1.5, 2.2, 2.4 (tested at 1.0, 2.5, 5.0)	1.7, 1.8, 4.7 (tested at 1.0, 2.5, 5.0)[e]	▪ PS reference data obtained from 2 studies ▪ known human sensitizer, species specific mechanism
Salicylic acid	0.8, 1.5, 2.5 (tested at 5.0, 10.0, 25.0)	2.3, 3.5, 9.5 (tested at 5.0, 10, 25)[e] 1.4, 1.5, 3.8 (tested at 5.0, 10, 25)[g]	▪ PS reference data obtained from 1 single study ▪ PS LLNA borderline negative ▪ insufficient human epidemiologic evidence for classification as sensitizer

Table adapted from Kolle et al., 2013 Regul Toxicol Pharmacol. 65(2):278-85.
[a] Underlined substances should be classified as skin sensitizers based on human evidence and previous LLNA
[c] Expressed as the stimulation index (SI) to one significant figure
[d] Values taken from the PS documentation (ICCVAM, 2009)
[e] Concurrent LLNA as published by Basketter et al., 2012 J Appl Toxicol. 32(8):590-6
[f] A concentration of 75% gave an SI value of 2.1
[g] Concurrent LLNA conducted as confirmatory study in a contract research laboratory in a blinded manner.

Corrositex® Proficiency Chemicals (OECD TG 435)

The Corrositex *In Vitro* Membrane Barrier Test Method for Skin Corrosion uses the breakthrough time of an artificial biomembrane to identify substances corrosive to the skin. To demonstrate technical proficiency the set of PC were assessed and the expected result of one PC was difficult to reproduce in three laboratories.

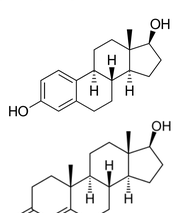
Lab/ run	Mean Breakthrough time [min]	Predicted UN GHS Classification
Reference data	61	1C
BASF run 1	44	1B
BASF run 2	54	1B
IIVS	43	1B
CRO[a]	55	1B

[a] Conducted as confirmatory study in a contract research laboratory in a blinded manner.

The mean breakthrough times observed for **tetraethylenepentamine** were close to the cut-off for GHS category 1C (i.e., > 60 min) in two runs at BASF, one run at IIVS and one run in a CRO. Numerical reference data (including the values for the four replicate vials) are difficult to find. Available *in vivo* reference data are not sufficient to derive a subclassification (references available upon request). Further *in vitro* data, including those from a CRO that has tested the identical material as the BASF lab, indicate that tetraethylenepentamine should probably be classified as GHS category 1B and is therefore not a good PC.

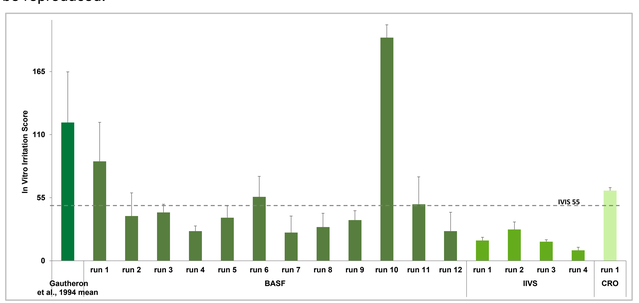
Steroidogenesis Assay Proficiency Chemicals (OECD TG 456)

The Steroidogenesis Assay uses a human adreno-carcinoma cell line that is competent in synthesising 17β-estradiol and testosterone. Concentrations of those hormones are measured after test substance exposure to evaluate whether a test substances affects steroidogenesis. While the majority of PC are readily available, the only negative PC, **human chorionic gonadotropin (hCG)** could not be tested due to the prohibitive costs and/or insufficient purities and its molecular weight.



BCOP Proficiency Chemicals (OECD TG 437)

The Bovine Cornea Opacity and Permeability test (BCOP) is an organotypic assay regulatorily accepted to identify seriously eye damaging (UN GHS category 1) and non-eye-irritating substances. To demonstrate technical proficiency the set of PC were assessed and the expected result of one PC (**dibenzyol-L-tartaric acid**) could not be reproduced.



There is *in vivo* evidence confirming UN GHS category 1 classification of dibenzyol-L-tartaric acid (references available upon request). The substance was correctly predicted during the establishment and in-house validation of the BCOP test (BASF run 1). Notably the results were not reproduced in the succeeding runs (BASF runs 2-9), with one run borderline positive with a large standard deviation. Three additional runs were conducted at BASF (runs 10, 11, and 12), all with the identical test substance batch. Run 10 was conducted applying neat dibenzyol-L-tartaric acid and resulted in an IVIS way above the cut-off value. Runs 11 and 12 were conducted with the 20% suspension but in run 11 an ophthalmic swipe was used to remove test substance residues while corneas in run 12 were washed using the OECD test guideline method. Of note one of the BCOP pioneer labs, IIVS, was also not able to correctly classify this substance in the BCOP (IIVS run 4 was conducted with the identical test material as BASF runs 1-4). Further, the test material used in BASF runs 10-12 was sent (blinded) to a CRO for confirmatory testing. The material was tested there as a 20% suspension after difficulties with the test substance preparation were reported.

CONCLUSIONS

- If the performance standards (PS) chemicals and proficiency chemicals (PC) are not chosen wisely, e.g. without sufficient experimental evidence for their inclusion, both test method evaluation and transferability can be hampered.
- When inappropriate reference chemicals are identified, these substances should be removed and replaced with chemicals with better reference data.

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