Optimization of the Direct Peptide Reactivity Assay (DPRA) for poorly soluble substances

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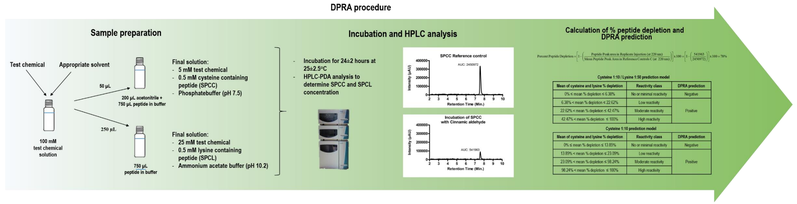
1 BACKGROUND AND OBJECTIVES

Assessment of the skin sensitization potential represents an important component of the safety assessment of chemicals used in consumer products. The Direct Peptide Reactivity Assay (DPRA) addresses the molecular initiating event of the skin sensitization Adverse Outcome Pathway (AOP), i.e. the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins. To quantify the reactivity of chemicals, model synthetic peptides containing cysteine (SPCC) or lysine (SPCL) are incubated with test chemical at a 1:10 and 1:50 ratio, respectively. After incubation, peptide depletion is determined and used in a prediction model to categorize the test chemical in one of four classes of reactivity to support discrimination between sensitizers and non-sensitizers.



When testing chemicals that show poor aqueous solubility, i.e. when precipitation is observed, it is uncertain how much of the test chemical is in solution to react with the peptide, and peptide depletion may be underestimated. In such a case a positive result can still be used, but a negative result is unreliable since a false negative result cannot be excluded.

Therefore, it is very important that a test chemical is completely in solution, especially in the case when a test chemical is negative in the DPRA. As such, selection of a suitable solvent is essential.

The objectives of the present study are therefore:

- Testing the effects of various solvents on peptide stability (i.e. the impact on SPCC and SPCL).
- Investigating the application of surfactants in the DPRA procedure to improve solubility of poorly water soluble substances (substances with a Log K_{ow} >4).

The OECD test guideline for the DPRA (TG 442C) mentions several suitable solvents. However, other solvents may be used as long as they do not impact on the stability of the peptides (< 10% peptide depletion after 24 hours of incubation).

To improve solubility and thus to prevent false negative results and to support true negative results, we investigated the feasibility of applying surfactants in the DPRA incubation procedure. Initially we investigated the effects of various non-ionic surfactants in the DPRA, followed by determination of the concentration of surfactant that showed no effects on the test system.

As a case study we investigated the applicability of hexaethylene glycol monododecyl ether ($C_{12}EO_6$), a non-ionic surfactant, to solubilize paraffinic substances in the DPRA procedure. These paraffinic substances comprised complex aliphatic mixtures of unbranched, branched and cyclic hydrocarbons and were observed to be negative in the DPRA earlier. However, since these substances have a high Log K_{ow} they are poorly soluble/insoluble in an aqueous environment (see Table 1) and thus a false negative result cannot be excluded.

Paraffinic substance	Estimated Log K_{ow}
Hydrocarbons, C8-C11, n-alkanes, isoalkanes, <2% aromatics	4.84
Hydrocarbons, C9-C12, n-alkanes, isoalkanes, <2% aromatics	5.48
Hydrocarbons, C12-C15, n-alkanes, isoalkanes, <2% aromatics	7.23
Hydrocarbons, C18-C24, isoalkanes, <2% aromatics	11.59

Table 1 Alkane composition and estimated Log K_{ow} of 4 paraffinic substances.

2 EFFECTS OF SOLVENTS ON PEPTIDE DEPLETION

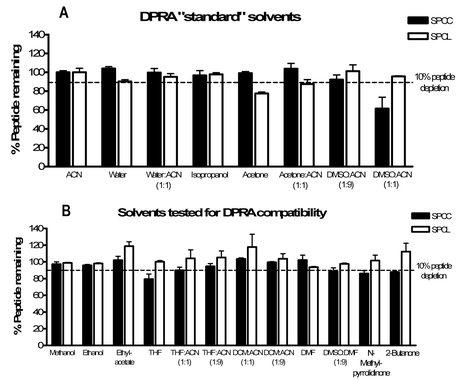


Figure 1 Effects of solvents on % SPCC and SCPL peptide depletion.

OECD Test guideline 442C mentions several suitable solvents for use in the DPRA. From these solvents (Figure 1A), acetone and DMSO:Acetonitrile (1:1, v:v) showed an effect on SPCL or SPPC peptide stability (>10% peptide depletion), respectively. As a result it is not recommended to use these solvents in the DPRA.

Testing showed that various other solvents were compatible with the DPRA (Figure 1B). However, some solvents showed not to be compatible with the DPRA since they caused depletion of SPCL (THF, N-Methyl-pyrrolidone and 2-Butanone), or an increase in SPCL signal due to interference with the analytical method (Ethylacetate, Dichloromethane:Acetonitrile (1:1, v:v) and 2-Butanone).

3 RESULTS

Application of surfactants in the DPRA procedure to improve solubility of poorly water soluble substances:

A standard DPRA assay was performed to determine the peptide reactivity of Tween 20, Tween 80, C12EO6, Brij O20, Brij 58 and Brij S100. All these surfactants were dissolved in Acetonitrile at a 100 mM concentration before application to the incubation solutions. The % peptide depletion, DPRA prediction and reactivity classification are presented in Table 2. The reactivity classification was performed using the Cysteine 1:10 / Lysine 1:50 prediction model.

Surfactant	% SPCC depletion (mean ± SD)	% SPCL depletion (mean ± SD)	Mean of SPCC and SPCL depletion (%)	DPRA prediction	Reactivity class
Tween 20	9.4 ± 0.4	1.1 ± 0.9	5.2	Negative	Minimal
Tween 80	15.3 ± 1.0	0.8 ± 1.1	8.1	Positive	Low
$C_{12}EO_6$	7.1 ± 0.5	0.0 ± 0.0	3.6	Negative	Minimal
Brij O20	22.2 ± 6.6	0.0 ± 0.0	11.1	Positive	Low
Brij 58	0.3 ± 0.6	0.0 ± 0.0	0.2	Negative	Minimal
Brij S100	2.1 ± 1.8	1.8 ± 2.6	1.9	Negative	Minimal

Table 2 Results of 6 non-ionic surfactants obtained in the DPRA.

Tween 20, $C_{12}EO_6$, Brij 58 and Brij S100 were negative in the DPRA, while Tween 80 and Brij O20 were positive in the DPRA and classified in the low reactivity class. All surfactants showed clearly no SPCL depletion. However, Tween 20, Tween 80, Brij O20 and in a lesser extend $C_{12}EO_6$, clearly showed depletion of SPCC.

As a follow-up dose response curves were generated to determine the surfactant concentration where no depletion of SPCC and SPCL was observed anymore. As examples the results for $C_{12}EO_6$ and Brij 58 are shown in Figure 2.

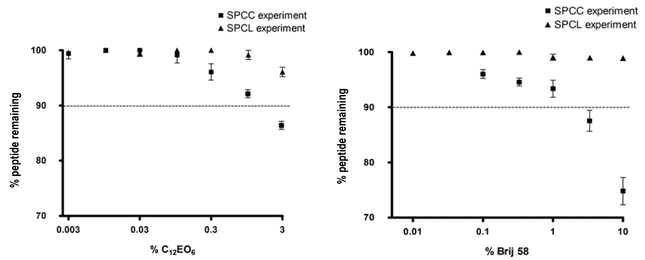


Figure 2 Effects of $C_{12}EO_6$ and Brij 58 on SPCC and SPCL peptide depletion.

At the highest concentrations of $C_{12}EO_6$ and Brij 58, clearly SPCC peptide depletion was observed. No concentration dependent effects of $C_{12}EO_6$ and Brij 58 on SPCL were observed.

Based on these results, the DPRA procedure was adapted and 0.05% $C_{12}EO_6$ was included in the phosphate or ammonium acetate buffer of the SPCC or SPCL incubation samples. Under these incubation conditions three (C8-C11, C9-C12 and C12-C15) out of the four paraffinic substances were dissolved completely.

The % SPCC and SPCL depletion, DPRA prediction and reactivity classification obtained for the four paraffinic substances in the adapted DPRA procedure are presented in Table 3.

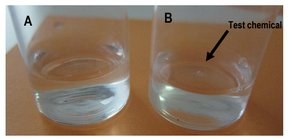


Figure 3 SPCC incubation mixture for a paraffinic test substance comprising of hydrocarbons, C_9-C_{12}, n-alkanes, isoalkanes, <2% aromatics) with (A) and without (B) 0.05% $C_{12}EO_6$

Paraffinic substance	% SPCC depletion (mean ± SD)	% SPCL depletion (mean ± SD)	Mean of SPCC and SPCL depletion (%)	DPRA prediction	Reactivity class
C8-C11, n-alkanes, isoalkanes, <2% aromatics	0.0 ± 0.0	1.2 ± 1.9	0.6	Negative	No or Minimal
C9-C12, n-alkanes, isoalkanes, <2% aromatics	0.0 ± 0.0	1.8 ± 3.1	0.9	Negative	No or Minimal
C12-C15, n-alkanes, isoalkanes, <2% aromatics	0.4 ± 0.7	0.7 ± 0.7	0.5	Negative	No or Minimal
C18-C24, isoalkanes, <2% aromatics	0.2 ± 0.4	0.1 ± 0.1	0.1	Negative, but due to insolubility results should be interpreted with due care.	

Table 3 Results obtained for paraffinic substances in the DPRA with inclusion of 0.05% $C_{12}EO_6$.

4 CONCLUSIONS

Effects of solvents on peptide stability:

- Not all solvents mentioned in the DPRA OECD test guideline were found to be compatible with the DPRA procedure: acetone and DMSO:Acetonitrile (1:1, v:v) showed >10% SPCL or SPCC depletion, respectively.
- Various other solvents not mentioned in OECD TG 442C showed to be compatible with the DPRA and can be added as alternatives in the DPRA solubility assessment procedure.

Application of surfactants in the DPRA procedure to improve solubility of poorly water soluble substances:

- Surfactants such as $C_{12}EO_6$ have the potential to solubilize poorly soluble substances (Log K_{ow} in the range of approximately 4 to 8) in the DPRA and thus can support the reliability of a negative outcome in the DPRA.
- The DPRA method with inclusion of a surfactant should be validated with inclusion of proper controls such as compounds that are known to be false negative in the DPRA due to incomplete solubilisation.