

Letter to the Editor

Reactivity assays – dealing with substances and samples in an *in vitro* world – the solution and the real problem

Dear Sir,

Reactivity assays (as reviewed in Ref. 1) of various designs are now under development for future use in novel non-animal approaches to predict skin sensitization potential. Recently, in *Contact Dermatitis* (2009; 61(5): 310–311), Roberts and Basketter posed in their Letter to the Editor some of the pertinent considerations around the use and interpretation of *in vitro* peptide:chemical reactivity assays for decision-making in the context of skin sensitization. As they stated, the purposes for predicting skin sensitization potential or the lack of it are:

- (1) for regulatory classification and/or labelling purposes;
- (2) for human safety assessment associated with product development;
- (3) for quality control or other purposes.

In all of the above and in many cases, we are often interested in *substances* that are not highly pure and can contain impurities. The major component X or an impurity in the substance can cause sensitization. In various circumstances, we may need to know (i) if the *substance* is sensitizing or (ii) whether a *component of the substance* is sensitizing.

In reactivity assays developed to date, peptides (of various designs) are incubated with a molar excess of substance. Peptide depletion values alone in such reactivity assays do not provide any mechanistic information and do not, therefore, indicate conclusively whether the major component X of interest in the substance is reacting (and hence causing depletion) or a minor impurity in the substance is causing the depletion. Roberts and Basketter propose that for *pure* substances or novel substances, where it is of interest to determine the sensitization potential of the major component X only, the incubation conditions should be switched and a molar excess of peptide nucleophile should be used. This would lead to the generation of two different experimental designs for reactivity assays depending upon purpose.

The authors of the letter appear to be working on the assumption that the only analysis that can be done within an existing reactivity assay is the

derivation of a peptide depletion value which can, in absolute terms, vary depending upon assay conditions. There is an alternative technical solution that would mean developing one reactivity assay design (i.e. building on the existing assays with substance in molar excess of peptide concentrations) but using different and concomitant back-end analysis depending upon purpose. The problem of determining whether the major component X or an impurity in a substance is causing peptide depletion can be determined by analysing the same sample (as used for peptide depletion analysis) for peptide-adduct identification using mass spectrometry in the assay design, as two groups have successfully achieved (2, 3). Recently, Natsch et al. (4) have shown that this can be done for the commercial fragrance chemical Azurone™ [7-isopentyl-2H-benzo(b)(1,4)dioxepin-3(4H)-one] empirically, and without any prior knowledge of a chemical's ability to react, yielding useful knowledge about mechanism of action.

What Roberts and Basketter propose (in reversing the incubation ratios so there is an excess of nucleophile to test substance) is a valid and cheap solution for screening *pure* substances, but is still not without ambiguity and limitations, and there is a lack of mechanistic interpretation. It is worth acknowledging the significant impact of the continuous and rapid improvement of analytical instrumentation, with specific reference to higher sensitivity limits of detection and increased dynamic range of mass spectrometers, in enabling us simultaneously to investigate and characterize both very low-level and high-level peptide adducts (i.e. impurities derived reaction products and pure chemical peptide adducts) in a single sample/experiment and with a high degree of reproducibility. Within a decade, such techniques can be expected to be accessible and routine.

A method incorporating a mass spectrometric analysis to identify peptide adducts enables unambiguous knowledge about mechanism of action to be generated that allows for more targeted risk management measures to be put in place using this knowledge, e.g. labelling of substance, preventing exposure to a specific chemical in factory settings,

removal of sensitizing impurities in supplies of substances, preventing oxidation in products, or reducing levels of the sensitizing component in the substance of interest to acceptable levels expected not to cause sensitization. There is also a great advantage in using mass spectrometry as an empirical tool in reactivity assays in that new mechanisms can be revealed that may not have been predicted by chemistry theory alone, or reveal multiple reactive pathways acting simultaneously, and increases confidence in identifying conclusively the specific reactive chemical(s) in a substance.

The importance of developing assays in this area is that they should be underpinned by the hypothesis of covalent chemical reactivity with proteins as a key step in sensitization, and knowing exactly which chemical species in the substance/sample has covalently reacted with the peptide is an important measure. It should also be noted that peptide depletion can be artefactual and not always caused by covalent reaction but by side reactions of oxidation, etc. Again, mass spectrometric analysis of adducts solves this problem.

The greatest issue facing the development of reactivity assays for sensitization prediction is determining which, if any, quantitative parameter(s) from this type of assay is/are most relevant towards the prediction of sensitization potency. As peptide depletion values can vary according to assay conditions and peptide:chemical ratios, it may be of more relevance perhaps to seek more 'universal'

parameters from these assays, e.g. reactivity rate constants through kinetics evaluations or extent of promiscuity of binding to different peptide-based nucleophiles (e.g. that could in principle lead to greater chances of antigen formation). Interpretation of peptide depletion values should be done with great care in attempting to make any hypothesis driven correlation with sensitization potency. A yes/no 'it is reactive' call may be all that can be derived from such a depletion measure alone.

Camilla Pease, Emma Thain and Raniero Zazzaroni
Unilever Safety & Environmental
Assurance Centre,
Sharnbrook, Bedfordshire MK44 1LQ, UK
e-mail: camilla.pease@unilever.com

References

1. Gerberick F, Aleksic M, Basketter D et al. Chemical reactivity measurement and the predictive identification of skin sensitizers. The report and recommendations of ECVAM Workshop 64. *Altern Lab Anim* 2008; 36: 215–242.
2. Natsch A, Gfeller H. LC-MS-based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. *Toxicol Sci* 2008; 106: 464–478.
3. Aleksic M, Thain E, Roger D, Saib O, Davies M, Li J, Aptula A, Zazzaroni R. Reactivity profiling: covalent modification of single nucleophile peptides for skin sensitization risk assessment. *Toxicol Sci* 2009; 108: 401–411.
4. Natsch A, Gfeller H, Emter R, Ellis G. Use of in vitro testing to identify an unexpected skin sensitizing impurity in a commercial product: a case study. *Toxicol In Vitro* 2009; October [epub ahead of print]. PMID: 19883749 (Pubmed).