

Review Article

Identification and classification of skin sensitizers: identifying false positives and false negatives

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The first step in regulatory evaluation of substances involves the identification of their intrinsic hazards, including the potential for skin sensitization. This is, quite properly, entirely different from assessment of the risks to human health, which might arise from incorporation of substances in products. EU guidance on regulations concerning the classification of skin sensitizers suggests a range of sources of information be deployed in the hazard identification process. These include chemical structure, predictive animal tests, and various types of human data. Where the information is clear-cut, then uncertainties rarely arise. However, for some materials, discordant information arises, perhaps because the substance is on the borderline of test sensitivity and classification (sensitizing materials of insufficient potency do not classified according to the EU scheme), due to conflicting results in predictive tests or for other reasons. In this study, we review data on a number of substances where a classification decision is complicated by such discordances and seek to use these examples to demonstrate how best to make a weight of evidence decision on whether a substance should, or should not, be classified as a skin sensitizer.

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The development of the EU required a harmonization of many things, including legislation in member states concerning the identification of the hazardous properties of substances (1). For example, for new chemicals introduced onto the European market, the Dangerous Substances Directive stipulates the information/testing required to demonstrate the presence or absence of a broad range of toxicological hazards, including that of skin sensitization (2). Harmonized guidance on deriving the appropriate classification and labeling for a substance is also available (3). For skin sensitizing substances (which would have the potential to cause allergic contact dermatitis (ACD) in sufficiently exposed individuals), the current criteria, originally established in 1996, are set out below:

Sensitization by skin contact

Substances and preparations shall be classified as sensitizing and assigned the symbol 'Xi', the indication of danger 'irritant' and the risk phrase R43 in accordance with the criteria given below:

R43: may cause sensitization by skin contact

- if practical experience shows the substance or preparation to be capable of inducing a sensitization by skin contact in a substantial number of persons or
- where there are positive results obtained from an appropriate animal test.

Human evidence

The following evidence (practical experience) is sufficient to classify a substance or preparation with R43:

- positive data from appropriate patch testing, normally in more than 1 dermatological clinic
- epidemiological studies showing ACD caused by the substance or preparation. Situations in which a high proportion of those exposed exhibit characteristic symptoms are to be looked at with special concern, even if the number of cases is small, or
- positive data from experimental studies in humans.

The following is sufficient to classify a substance with R43 when there is supportive evidence:

- isolated episodes of ACD or
- epidemiological studies where chance, bias, or confounders have not been ruled out fully with reasonable confidence.

Supportive evidence may include the following:

- data from animal tests performed according to existing guidelines, with a result that does not meet the criteria given in the section on animal studies but is sufficiently close to the limit to be considered significant,
- data from non-standard methods, or
- appropriate structure–activity relationships.

Animal studies

- Positive results from appropriate animal tests: in the case of the adjuvant-type test method for skin sensitization is detailed in Annex V (the guinea-pig maximization test) or in the case of other adjuvant-type test methods, a response of at least 30% of the animals is considered positive.
- For any other test method a response of at least 15% of the animals is considered positive.

In addition to the above, there is a short note that indicates that substances that cause immunological contact urticaria, but which are not classified as respiratory sensitizers, should be considered for classification with R43. It is also worth noting that because these criteria predate the formal validation and acceptance of the local lymph node assay (LLNA), there is no specific wording that refers to this method, although as an official Organisation for Economic Cooperation and Development (OECD) Test Guideline (number 429), it is a fully accepted alternative in the EU for making positive and negative classification decisions (4–6).

Considering the above EU classification criteria for skin sensitization, it is obvious that situations will arise where the evidence is either difficult or contradictory. For example, weak sensitizers may give results close to the classification borderline, being just above the limit in one test and just below it in another; some substances may fail to classify, giving below classification limit responses in predictive tests but then may show some evidence of human sensitization; occasionally, interspecies differences, e.g. in bioavailability/metabolism, may result in a substance being very clearly positive in one species but entirely negative in another. Of course, it is important to remember that human evidence has to be considered in the sense of how

it informs us about the presence/absence of hazard; EU classification of dangerous substances is hazard-based legislation although an expert group under the auspices of the European Chemicals Bureau recently concluded that the results from the routinely used hazard sensitization test may be evaluated more sensibly by taking into consideration dose–response relationships (7). Ultimately, all the information on a substance must be weighed to lead to a balanced hazard classification decision. In this study, a number of examples are given with the aim of illustrating the points and so providing guidance. It should be noted that the authors have not compiled here exhaustive reviews of all the data for the substances selected but rather compiled results that are representative and serve to illustrate the conclusions drawn.

Materials and Methods

The substances chosen are sodium lauryl sulfate (SLS), cocamidopropyl betaine (CAPB), sulphamonic acid, benzalkonium chloride (BKC), propyl/butyl paraben, limonene, and methylidibromoglutaronitrile (MDGN). Data are taken exclusively from published literature, or material in press, but deliberately does not represent an exhaustive search. The information sets have then been considered in relation to the EU classification criteria given above. It should be noted that EU classification decisions on these substances would have access to unpublished data, largely from producing industries and so might differ from classification decisions here. The aim of this study is to exemplify the problems and provide suggestions for guidance, not to argue for particular classification outcomes for the particular substances herein or to indicate potential safe thresholds, either for induction or for elicitation of ACD. In all these considerations, note has been taken of the current wording of legislation (3), technical guidance documents (8), and published expert opinion (9–11).

Results

Propyl/butyl paraben

The family of paraben preservatives do cause ACD in humans (12, 13) and thus must be skin sensitizers. However, should they be formally classified as significant skin sensitizers? They are negative in predictive animal assays, both guinea-pig and mouse and thus might be regarded as very weakly sensitizing (14). Such a conclusion in fact fits with the clinical data because despite very widespread human exposure, e.g. via leave-on cosmetics, the only situation where there is any frequency of ACD to this preservative family is

with medicament exposure, typically in association with stasis ulcers, giving rise to the term ‘the paraben paradox’ (15). Relatively recently, Wilkinson and colleagues reviewed preservative allergy in Europe, including testing of the paraben mix at 16% in petrolatum (approximately 50 × the normal maximum use concentration). The percentage of positive patch tests was steady at 0.6–0.9% over the 10 year study period and was the lowest of the 7 preservatives studied (16). In a more recently published European survey of more than 26 000 patients, the percentage of positive patch tests remained at the average at 0.6% (17). Nevertheless, in normal use, the paraben preservatives are very rare contact sensitizers and ‘set the standard for relative safety that new preservatives will have difficulty matching’ (12). Therefore, in terms of regulatory classification, the most sensible conclusion would be that while sensitizing, paraben preservatives possesses this property to such a limited degree that formal classification is not warranted.

Sulphanilic acid

This substance is very clear positive in the guinea-pig maximization test (100% of the animals were sensitized) but negative in the LLNA at an exposure concentration of up to 10%, at which level the stimulation index had reached only 1.5 (it must reach 3 to be regarded as indicative of skin sensitization) (18). To try to understand which of these results was most reflective of the true human situation, the same publication then considered the occupational exposure to the chemical. The absence of any evidence of ACD to sulphanilic acid over >20 years in the exposed workforce (amongst which no specific precautions to avoid skin exposure were in place) led to the conclusion that sulphanilic acid was not an important skin sensitizer and thus would not merit formal classification (18).

Benzalkonium chloride/sodium lauryl sulfate

A classic skin irritant chemical (R38), such as BKC or SLS, will be likely to present the risk of producing false positives in both predictive animal tests for sensitization hazard identification and diagnostic assessments in humans (19). This does not mean that there is never real evidence of skin sensitization, but it does suggest that a careful assessment of positive results is required both for predictive tests in the guinea-pig (12, 13) and the mouse (19) and for diagnostic tests in patients with eczema. As has already been shown, it is all too easy to become confused and regard a material of this type as an important skin sensitizer, whereas in

reality, these materials should not be classified as R43, only as irritants, e.g. with R38 (20, 21).

Cocoamidopropylbetaine

CAPB represents an interesting and indeed complex problem in terms of regulatory classification. The published skin sensitization data show evidence of sensitization in both guinea-pig (22) and murine assays (23). This evidence would be sufficient to merit an R43 classification. In general, support of this is the evidence that CAPB can cause ACD, even if not to a great degree (24, 25). An essential consideration here is that exposure most commonly occurs from products that do not deliver prolonged skin exposure, e.g. shampoos, but, nevertheless, serves to confirm that the hazard with CAPB exists. However, over a number of years, work has repeatedly indicated that CAPB is not actually the allergen, but it is in reality two ‘impurities’, dimethylaminopropylamine and cocoamidoamine, which are responsible (26, 27). These impurities are in fact a starting material and a manufacturing intermediate, respectively. The consequence is that their levels can be controlled and by doing so leads to a CAPB supply that can be demonstrated to be safe in use, even for individuals who are already sensitized (23, 28). Thus, the balance of judgement in this case should be that CAPB (manufactured properly to a suitable purity specification) does not require formal R43 classification. This leads to discussion of another interesting material, limonene.

Limonene

Limonene is a flavour/fragrance chemical. In predictive animal assays, it is, at most, weakly sensitizing, producing results that might well be regarded as insufficient to trigger classification (29, 30). Clinically, it was not known to be a significant cause of ACD (31). However, once an awareness began to develop that air oxidation could lead, readily, to the formation of sensitizing impurities, perspectives on this substance began to change and R43 classification was thought more appropriate (29, 32). The evidence that diagnostic patch testing with oxidized limonene led to a ready identification of sensitized individuals lent credibility to such a point of view (33). Then, given that air oxidation may be hard to avoid, it is possible to conclude that R43 is appropriate for limonene.

Methyldibromo glutaronitrile

MDGN (also known as dibromodicyanobutane and often under the trade name Euxyl K400) is

a preservative. Earlier published studies on the guinea-pig indicated that this substance was not a significant skin sensitizer (34, 35). Subsequently, human clinical data contradicted these observations, suggesting that MDGN was capable of inducing skin sensitization, and although earlier data were associated with somewhat unusual uses (e.g. moist toilet paper) (36), overall, the picture increasingly has been of a substance that clearly possessed significant skin sensitization potential (16, 37). Here, most notable was the work of Wilkinson and colleagues who collated data over a 10 year period for many European clinics and demonstrated a sharp rise in the frequency of positive patch test reactions to MDGN (16). Many of the cases of sensitization to MDGN have been demonstrated to be associated with normal cosmetic and occupational use of this preservative (38, 39). Even the short-term exposure associated with repeated use of liquid soaps preserved with MDGN is a significant cause of MDGN allergic dermatitis (40). In addition, more recent LLNA data indicated a clearly positive result, consistent with the material being regarded as a strong sensitizer (41). Thus, MDGN would be judged correctly to be a classifiable skin sensitizer, and based on the available evidence, the EU Scientific Committee on consumer Products concluded that as no safe use level for MDGN in rinse off product has been established, it is recommended that it should be present in any cosmetic products (42).

Conclusions

Toxicology regulations on substances quite rightly need to focus on hazardous properties, not on risk to human: hazard is a property of the substance; risk to human arises from inappropriate exposure to this hazard. Legislation on preparations incorporating such substances will obviously need to take the opposite perspective, risk to human health not hazard (43). In the EU, the first element is properly dealt with as the primary focus is on hazard identification. Nevertheless, there is a caveat, inasmuch as hazardous properties are often not 'all or none' phenomena, and this is the case for skin sensitization and is also accommodated to some extent in the legislation; many substances are sensitizers, but for a proportion, they possess this characteristic to such a limited degree that formal classification would be entirely inappropriate. This was recognized from the outset in EU legislation by the incorporation of classification thresholds into the (then) existing animal predictive tests (1). Subsequently, it was also recognized in the updates to classification criteria, e.g. by accepting that positive classification

using human data might only be allowed where there were multiple cases of allergy from more than 1 clinic (3).

The practical outcome of the above is that the existing EU classification scheme involves consideration of the potency of sensitization, rather than just its presence. Importantly, it is also based on the frequency with which skin sensitization effects are seen, not at all on the severity of reactions seen in an individual, the latter being a risk-based measure, dependent on individual susceptibility and exposure considerations. The practical outcome of this is that classification decisions are more complex because rather than an R43 being awarded on the basis on any credible evidence of skin sensitization, it requires a sufficient weight of credible evidence. 'Weight of credible evidence' is inevitably a matter of expert judgment and other factors, some political and some scientific. Such is the topic of this article.

As already mentioned earlier, there would be little argument that a strongly sensitizing chemical such as *p*-phenylenediamine would be classified because it causes very evident sensitization in both predictive animal models and human (44). A similar argument might be made for glycerol as a substance that should not be classified and which can be safely deployed in the treatment of dry skin and atopic dermatitis (41, 45). The challenge comes with substances that are closer to the borderline, and these are the ones discussed here. A chemical that is strongly positive in predictive assays normally would not command discussion. The situation with sulphanilic acid suggests, however, that this might not always be the case as strong evidence of sensitization in the older standard, the guinea-pig maximization test, could be overruled by data from the LLNA and from human experience. In this case, it is the human experience that, in our view, tips the balance in favour of non-classification (18).

It is worthwhile to consider what really represents good human experience, suggesting an absence of hazard rather than just risk. In this judgment, it is exposure that is critical; water represents a major inhalation hazard if the dose is high enough but to conclude that because there is no report of anyone drowning during a rain shower that water is not an inhalation hazard would clearly be incorrect. So, evidence of an absence of a sufficient sensitization hazard to trigger classification requires, in our view, knowledge of extensive skin exposure over several years to large numbers of people. Quantification of these numbers is not really possible; data must be judged on a case-by-case basis in the light of the other available information. The sulphanilic acid

case is, in a sense straightforward, because, being so strongly positive in one test and so clearly negative in another equally valid assay, simply required some other information to tip the balance. It contrasts to a certain extent with the case of BKC. Here, various limited pieces of evidence combine to make a weak case for classification and what argues against this is the view that the human evidence is exaggerated (20) as well as being modest in quantity. A better comparison, however, occurs with MDGN, where despite limited historical predictive test data originally indicating no significant degree of sensitizing activity, the clear conclusion from human exposure, as well as more recent LLNA data, must be that an R43 classification is warranted (36–40). An issue, here, that is troubling is why the predictive test data from the earlier study were incorrect. Technical difficulties in the conduct of guinea-pig predictive tests may be part of the reason.

One final issue raised in this review is the question of impurities/oxidation products. For limonene, the EU regulatory view was that avoidance of the formation of sensitizing species as a result of oxidation was unavoidable, and hence, R43 classification was appropriate. This might well contrast with CAPB, where the presence of sensitizing impurities arises within the manufacturing process. Control of this process can lead to high-quality material that is, to all intents and purposes, non-sensitizing (28). Perhaps, in the future, it might be possible to add considerations of purity specifications into the regulatory thinking, not least as a driver of the quality grade of chemicals that are unlikely to lead to the induction of skin sensitization.

False-positive and false-negative data are an inevitable consequence of predictive toxicology methods, which, whether *in vivo*, *in vitro*, or *in silico*, are unlikely ever to be 100% sensitive and 100% specific. This is certainly the case for skin sensitization. As a consequence, the final test of whether a chemical is truly a skin sensitizer must come from human experience. Because the human experience will almost always comprise ad hoc information, rather than from well-controlled clinical studies, then expert judgement will inevitably be necessary. The examples above it is hoped will provide some potential benchmarks that might be used to facilitate such judgements.

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