

An interlaboratory evaluation of the Buehler test for the identification and classification of skin sensitizers

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The correct identification of potential skin sensitizers is an essential first step in enabling a proper risk assessment to be made and to permit the implementation of appropriate risk management practices designed to avoid the induction of sensitization. Consequently, regulatory guidelines around the world demand that new substances are evaluated to assess their skin sensitization potential. There are two guinea pig test methods which are generally recognised, the guinea pig maximisation test (GPMT) and the occluded patch test described by Buehler. In different countries, one procedure seems to be more prevalent and acceptable to regulatory authorities than the other. Notably, in the European Union, the latest revision of the Annex V (Directive 92/32/EC) Test Method for skin sensitization asks that justification should be given in the situation where the notifier does not use the GPMT, which is the preferred method. Thus in this paper, the validity of the Buehler protocol in the context of European legislation is critically examined. Results from two laboratories are collated, showing that the method can identify significant contact allergens, particularly those which would be registered formally as such according to European legislation. It is demonstrated that minor methodological variations can be tolerated without compromising test sensitivity, but it is recommended that suitable positive control testing is the best way to ensure proper test conduct.

Key words: Buehler test; guinea pig sensitization; allergic contact dermatitis; Annex 5 (directive 92/32/EC) Test Method; European Union legislation; interlaboratory evaluation; © Munksgaard, 1996.

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There has been debate over many years about which of the numerous skin sensitization tests conducted in experiments on animals are most suitable for determining inherent skin sensitization potential towards man. However, what is clear is that the correct identification of potential skin sensitizers is an essential first step in enabling a proper risk assessment to be made and that this then permits the implementation of appropriate risk management practices designed to avoid the induction of sensitization. Consequently, regulatory guidelines around the world demand that new substances are evaluated to assess their skin sensitization potential (1-4). The single most important criterion to meet is the ability to detect human contact allergens with suitable sensitivity and specificity. This is apparent in the various regulatory guidelines. Thus the Annex V of the EEC Directive for Dangerous Substances (92/32/EC) states "The sensitivity and ability of the test to detect potential human sensitizers are considered important in a classification system for toxicity relevant to public health" (1). The OECD Guideline on

skin sensitization has a different wording, but in essence describes the same criterion: "The test selected should be a reliable screening procedure which should not fail to identify substances with significant allergenic potential while at the same time avoiding false negatives" (2). Nevertheless it is important to note that no single test will be 100% predictive in identifying potential human skin sensitizers. The absence of an infallible skin sensitization test is also reflected in the EC directive (1) which states "There is no single test method which will adequately identify all substances with a potential for sensitizing human skin which is relevant for all substances".

In almost all regulations there are two test methods which are universally recognised, these being the guinea pig maximisation test (GPMT) (5, 6) and the occluded patch test described by Buehler (7, 8). However, as reported elsewhere (9), in some countries one procedure seems to be more prevalent than the other. Notably, in the European Union, the latest revision of the Annex V Test Method for skin sensi-

Table 1. Interlaboratory comparison of the Buehler test

Substance	Induction	Challenge	Result ¹		Induction	Challenge	Result ¹	
			Lab. 1				Lab. 2	
dinitrochlorobenzene	0.3%	0.2%	100%		0.3%	0.2%	100%	
dinitrochlorobenzene	0.3%	0.02%	50%		0.3%	0.02%	50%	
hexylcinnamic aldehyde	20%	2.5%	70%		50%	5.0%	60%	
eugenol	100%	30%	11%		75%	25%	0%	

¹ Results expressed as the % of the test group judged as having positive allergic reactions.

tization (1) asks that a justification should be given in the situation where the notifier does not use the GPMT, which is the preferred method. This places the European regulatory authorities in some difficulties, since the Buehler Test is a legitimate procedure within the OECD and thus they must accept the data from this method. However, they have a right to expect that the test is conducted properly and is demonstrably valid for the purposes of meeting European law. Thus in this paper, the validity of the Buehler protocol in the context of European legislation is examined critically and the key factors in ensuring that this test is adequately carried out are reinforced. In particular, we report historic results of the evaluation of 37 clinically known skin sensitizers. Data on known non-sensitizers has not been included since the issue is one of the sensitivity of the assay, but it should be noted that our experience is that, when properly conducted and controlled, false positives do not occur in the Buehler test.

Materials and Methods

The Buehler Test data in this paper are derived from the historic databases existing in the two laboratories (P&G=Laboratory 1; Unilever=Laboratory 2), supplemented by a limited amount of other published data. The protocol followed has been described in detail previously (10) and this was the method used by laboratory 1. The method applied by Laboratory 2 has been reported before (11) and differed from that of Laboratory 1 in only minor aspects, notably in the means of achieving proper occlusive skin contact with the test chemical during patch treatments. The potential impact of these differences can be minimised and is discussed later. For the sake of clarity in the present paper, the principal details of the Buehler tests are given below.

Buehler test method

The Buehler test protocol uses an occluded topical patch technique for induction and elicitation of contact sensitization (8, 12). The procedure calls for 20 test animals in the test group, 10 naive control animals for challenge and 10 separate naive

control animals for rechallenge. Three 6-h induction patches are all applied to the same clipped (shaved) induction site on the dorsal surface of the test animals, one patch/week for 3 weeks. The patches are securely occluded with the use of a restrainer (10) or the use of a wrapping technique (11). The induction sites can be graded for erythema during the 3-week induction period; however, depilation is not used prior to assessing induction erythema. Following a 10–14 day rest, the test animals receive 6-h challenge patches at a naive skin site. Ten naive control animals are challenged in an identical manner. Reactions are graded for erythema 24 h and 48 h after patch removal (with depilation 2–3 h prior to the 24 h scoring time point), according to erythema scale (0, 1, 2, 3). The grades "1", "2", and "3" denote increasing severity of erythema with grades $\geq$ "1" considered positive. A test chemical is considered positive in the context of European classification criteria, if an incidence of at least 15% is achieved in the test group (13). Test animals and the 10 new control animals can be rechallenged 7–15 days after primary challenge at any remaining naive skin sites.

Although the skin sensitization regulatory guidelines call for the use of 20 test animals in the Buehler test (1, 2), some of these investigative studies were performed with less than this number, usually 10. Details of the specific numbers involved can be found in the original references, but as the data show this was not an important variable.

Chemicals tested

The majority of substances chosen have been taken from existing publications from the two laboratories. For all of these and for those which are as yet unpublished, details are given in Tables 1, 2. The rationale for the choice of these substances is that they either would or would not classify as skin sensitizers according to European criteria (13) and that this classification is broadly in accord with human experience. The support for this judgement comes from Annex I of the European Commission which contains the existing classification data (14) or from the detailed review of suitable positive and negative

Table 2. Buehler guinea pig skin sensitization test results

Substance	Induction	Challenge	Result ¹	R43 ²	Reference
abietic acid	25%	25%	0%	—	27
allylthiocyanate	0.75%	0.75%	30%	+	8
ammonium thioglycolate	10%	5.0%	35%	+	28
amylcinnamic aldehyde	30%	10%	100%	+	28
benzocaine	50%	50%	20%	+	8
benzoyl peroxide	10%	10%	42%	+	23
chloramine T	2.5%	2.5%	70%	+	27
cinnamic acid	10%	10%	0%	—	28
cinnamic alcohol	30%	10%	50%	—	12
cinnamic aldehyde	10%	1.0%	80%	+	28
citronellal	2.5%	1.0%	45%	+	12
dansyl chloride	40%	5.0%	90%	+	8
D&C Yellow no. 11	40%	10%	69%	+	30
dinitrochlorobenzene	0.1%	0.1%	100%	+	31
dodecene unsaturated sulfone	0.35%	0.1%	87%	+	32
ethanol	80%	80%	60%	—	12
ethylenediamine	1.0%	0.25%	80%	+	29
eugenol	75%	25%	0%	—	27
formalin	5.0%	5.0%	30%	+	8
hexamethylene-1,6-diisocyanate	10%	1.0%	100%	+	28
hexyl cinnamic aldehyde	50%	5.0%	60%	+	11
hydroxycitronellal	30%	10%	25%	+	8
isononanoyloxybenzene sulphonate	25%	5.0%	90%	+	27
Kathon CG biocide	0.2%	0.2%	100%	+	33
2-mercaptobenzothiazole	75%	75%	55%	+	11
p-methoxycinnamic aldehyde	10%	1.0%	60%	+	28
monobenzylether of hydroquinone	5.0%	5.0%	60%	+	12
oxazalone	1.0%	0.1%	100%	+	29
p-phenylenediamine	10%	5.0%	90%	+	31
phenyl salicylate	25%	25%	0%	—	34
phthalic anhydride	25%	10%	30%	+	27
potassium dichromate	10%	3.0%	20%	+	28
tetrachlorosalicylanilide	1.0%	1.0%	80%	+	8
thioglycerol	14%	14%	60%	+	8
toluene-2,4-diisocyanate	10%	1.25%	95%	+	28
trimellitic anhydride	25%	10%	70%	+	27
vanillin	2.5%	2.5%	40%	+	12

¹ Results expressed as the % of the test group judged as having positive allergic reactions.

² Indicates whether according to EU criteria the chemical is, or would be, classified as a skin sensitizer.

control sensitizers, including those recently described by ECETOC, a European chemical industry group (15). In total, Buehler test data on 31 chemicals that are, or would be, classified as skin sensitizers and 6 not so classified has been collated.

Results

In Table 1, detailed results from the two laboratories have been presented in order to demonstrate inter-laboratory comparability. These are for a limited number of substances, including DNCB at the concentrations proposed by Buehler as a means to validate the quality of his procedure (16). In agreement with Buehler's findings, both laboratories found that the higher concentration of DNCB gave a 100% positive response, while the lower concentration gave a 50% response. Both laboratories obtained a

result with eugenol which would fail to classify according to EC criteria (13), whilst each laboratory obtained a very similar positive result with hexyl cinnamic aldehyde which was clearly well above the classification threshold. Of particular note is the eugenol data – it was totally negative in the test at Lab. 2, but gave a low level of response at the slightly higher concentrations used in the test at Lab 1. Thus it is interesting that eugenol is a known human skin sensitizer, but that it can be variable and very weakly sensitizing even in the guinea pig maximisation test (17).

Buehler test results with 37 substances are given in Table 2. (We have deliberately chosen not to readily identify from which of the 2 test laboratories the data originates, since this is not relevant to the matter in hand.) Of the 31 which would classify as skin sensitizers according to EC criteria, all 31 were pos-

itive at $\geq 15\%$, the threshold for classification defined by the EC for this method. Consistently reproducible positive results with eugenol have only been obtained with the murine local lymph node assay (18, 19). Of the 6 substances which would not be classified (although they may of course still be weak skin sensitizers), cinnamic alcohol and ethanol were clearly positive. These substances are recognised, but not particularly common, human contact allergens (20, 21).

Discussion

The identification of skin sensitizers is a critical first step in enabling a risk assessment to be made and suitable risk management practices implemented. Consequently, regulatory guidelines around the world demand that substances are evaluated to assess their skin sensitization potential (1–4). There are two test methods which are generally recognised, the guinea pig maximisation test (GPMT) (5, 6) and the occluded patch test of Buehler (7, 8, 12). As the protocol involves epicutaneous induction and challenge, the Buehler test is considered to be well suited to the evaluation of chemicals for which may be expected to come into contact with human skin, e.g., via occupational or consumer exposure (8, 22, 23). However, as reported elsewhere (9), in some countries use of the GPMT seems to be more prevalent and data from that test more readily accepted by regulatory authorities. Of particular note is that in the European Union, the latest revision of the Annex V Test Method for skin sensitization (1) asks that a justification should be given in the situation where the notifier does not use the GPMT, which is stated to be the preferred method. Thus we have chosen to examine critically the validity of the Buehler protocol in the context of European legislation and to reinforce the key factors in ensuring that this test is properly carried out.

Initially it has been demonstrated that the Buehler test can be carried out to a very similar standard in two laboratories (Table 1). It should be noted that the data presented is historic and even though the samples of the substances used were not the same and the studies were not done at the same time, the results were in close agreement. This argues for the robustness of the method when properly carried out. The main difference in test conduct was that Lab. 1 used restraint during animal treatment to ensure proper occlusion of the test sites, whilst Lab. 2 employed firm wrapping to clipped and razored skin sites to achieve the same effect. The data presented in Table 1 could be used by other laboratories who wish to carry out the Buehler test as a guide to the quality of results that need to be obtained. Further

details, e.g., on vehicles etc., are given in the appropriate references.

Table 2 then lists Buehler test results with 37 clinically known skin sensitizers, 31 of which would be expected to be (or already are) classified according to formal EC criteria. The historical Buehler test results clearly demonstrate the sensitivity and ability of the test to detect a wide range of known skin sensitizers representing various chemical classes. The great majority of these skin sensitizers, which would be formally classified as such according to current European Guidelines, gave a response $\geq 15\%$ (see Table 2). As expected, moderate to strong sensitizers (e.g., dinitrochlorobenzene, dodecene unsaturated sulfone, Kathon CG, oxazolone and p-phenylenediamine) were easily detected with the Buehler test.

Importantly, the test has demonstrated its ability over the years to detect chemicals with relatively weak potential to sensitize humans via patch test or in-use exposure (8, 12, 24). Table 2 lists several substances which would be considered weak sensitizers on the basis of published adjuvant test data (e.g., cinnamic alcohol, citronellal, ethanol). Such chemicals may often be sufficiently weak skin sensitizers that they would not be formally classified on the basis of European criteria. Of more significant note in the present context, the Buehler test has been demonstrated to be capable of detecting the 3 OECD recommended positive control substances with mild/moderate sensitization potential (2). The 3 positive OECD control sensitizers detected by the Buehler test are hexyl cinnamic aldehyde, mercaptobenzothiazole, and benzocaine (Table 2). The performance of the Buehler test compares favourably with the GPMT and a newer alternative (non-adjuvant) method, the local lymph node assay, in identifying these substances (11).

Now, European legislation is clearly aimed at identifying important, rather than all, skin sensitizers. This position is evident from the manner in which not only human evidence, but particularly animal data from prospective tests, is used for decisions on whether or not to classify a chemical as a skin sensitizer. Human data must be from a "substantial number of persons", whilst the animal data is subject to thresholds (30% positive for adjuvant tests; 15% positive for non-adjuvant tests) (13). Unequivocal evidence of the capacity of a chemical to cause skin sensitization, but which does not meet, or exceed, these thresholds, does not result in classification of that chemical as a skin sensitizer. In this way, the EC has put in place a pragmatic approach which it hopes will permit important human contact allergens to be identified, classified and suitably labelled, but which will not result in a large number of

minor sensitizers (and there are very many) also being classified, thus avoiding dilution of the impact of classification and the creation of unnecessary complications in risk management.

The data presented give confidence that the Buehler test identifies with sufficient sensitivity the degree of skin sensitization hazard which would lead to a chemical being formally classified according to EC criteria. However, to ensure this level of sensitivity, there are a number of important points of consideration for conducting a proper Buehler test. As stated previously, the test material needs to be properly occluded to the guinea pig skin. The definitive technique for assuring proper occlusion of the test material is the use of a device that prevents twisting or turning movements that could otherwise compromise the degree of patch occlusion obtained (10). However, another successful method employed to assure proper occlusion is the use of an adequate wrapping technique (e.g., elastic bandage) (11). Proper and tight occlusion of the patch ensures thorough contact of the skin with the test substance and allows the skin to hydrate which may enhance skin penetration of some materials (25, 26).

In order to optimise its sensitivity, the Buehler test should be conducted at the highest possible test substance concentrations consistent with its irritation profile (12). For induction, a dose should be chosen which produces mild to moderate irritation in the preliminary irritation studies. For challenge, the highest possible non-irritating dose is essential (1). Furthermore, the use of a proper vehicle is a critical factor to consider in designing a Buehler test for a particular substance. The most suitable vehicle is generally the one that best solubilizes the test substance at the desired test concentrations without producing vehicle irritation effects. If these steps are followed, it is our experience that the test can be conducted not only with great sensitivity but also with specificity; non-sensitizers are not found to produce false-positive results (data not shown).

It is concluded that the Buehler test can be conducted, with appropriate care, to exhibit both high sensitivity, in order to identify real skin sensitizers, and with high specificity, in order to discriminate the real non-sensitizers, such that the information provided satisfies the requirements for the EC classification of skin sensitization.

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