crystal violet (CV) method using transformed rabbit corneal (SIRC) cells (SIRC-CV); h) the NRU method using SIRC cells (SIRC-NRU); i) the reduction of 3-(4,5-dimethylthiazole-2-yl)-2-5-diphenyl tetrazolium bromide (MTT) using human cervical carcinoma (HeLa) cells (HeLa-MTT); j) the CV method using Chinese hamster lung (CHL) cells (CHL-CVS); and k) EYTEX.. A total of 27 laboratories across Japan participated in the validation study. Each method (except for MATREX and CHL-CVS) was assessed in at least five laboratories, and most laboratories assessed more than method.

The test chemicals, 38 cosmetic ingredients, were tested in three phases (9, 15 and 14 ingredients in the first, second and third phases, respectively). The samples were coded, randomised, supplied to the participating laboratories, and tested in accordance with the principles of GLP. The *in vitro* data were compared with Draize rabbit data obtained by a single laboratory in accordance with OECD Guideline 405, and the variabilities of the *in vitro* and the *in vivo* data were analysed.

The interlaboratory variability, as judged by the mean CV (the coefficient of variation averaged across all chemicals), was less than 50% for all tests except the HET-CAM and HD tests, for which the mean CVs were greater than 50%. However, the mean CVs of these tests could be brought into the same range by excluding the non-irritants from the analysis. The *in vivo* data were more variable, particularly in the range of MMAS values from 15-50, which is important for the evaluation of cosmetic ingredients. The correlation between the *in vitro* results and the MMAS was high (Pearson's coefficient greater than 0.7) for CAM-TB, HD, SIRC-CVS, SIRC-NRU, HeLa-MTT and CHL-CVS, but it was low (0.3) for EYTEX. The Pearson correlation coefficients for the cytotoxicity tests exceeded 0.8 if acids, alkalis and alcohols were excluded. The MMAS scores were also grouped into five categories according to the Kay and Calandra classification scheme (1962): non-irritant ($0 \leq MMAS < 0.5$), slight irritant ($0.5 \leq MMAS < 15$), mild irritant ($15 \leq MMAS < 25$), moderate irritant ($25 \leq MMAS < 50$) and severe irritant ($50 \leq MMAS$). The rank correlation between the *in vitro* results and these categories was high (Spearman coefficient greater than 0.8) for HET-CAM and CAM-TB, and was increased if powdered substances were excluded from the analysis. In the case of the cytotoxicity tests, the rank correlation was also greater than 0.8, provided that the acids, alkalis and alcohols were excluded. In general, the Spearman rank correlations were higher than the Pearson correlations. In addition to comparing the *in vitro* data with the MMAS, comparisons were also made with the 24h weighted Draize score and with individual tissue (cornea, conjunctiva and iris) scores. In general, however, stronger correlations were obtained using the MMAS. On the basis of these results, it was concluded that none of the alternative methods could be used to test all types of test substances, and that a battery of tests would be needed to optimise the predictivity of eye irritancy.

The Benchmarking/Reference Standards Approach

The term "reference standard" should not be confused with "positive control". A positive control is a substance which is known to give a positive response in a particular *in vitro* assay, and which is used to validate the correct conduct of the assay. In contrast, a reference standard (RS) is a substance which has a known degree of toxicity *in vivo*, and which can be used *in vitro* to determine the degree of toxicity of test substances, whose effects are scaled relative to the RS. For example, if two RSs were available corresponding to known boundaries of eye irritation potential (e.g. R41/R36 and R36/NI), it should be possible to classify a test

substance by comparing its *in vitro* result with the *in vitro* results of the two RSs. It should also be possible to obtain a measure of confidence for the classification according to the proximity of the *in vitro* result to each boundary. Conceivably, a positive control could also act as a reference standard, if it were used both to determine the validity of an assay and to scale the toxic response of a test substance.

In industry, RSs are already widely used for making safety decisions regarding the acceptability of new formulations of existing ingredients, and for prioritising further developments. Three other roles of RSs can be foreseen: a) within companies, for the development and cross-validation of *in vitro* assays - RSs could be used to investigate and calibrate new or existing assays by using data available in the public domain; b) in the validation of alternative methods, as a replacement for the totally blind approach which currently exists, so that substances can be grouped into categories defined by the RSs; and c) in regulatory toxicology, for the submission of data on selected new substances to competent authorities. This would apply to substances whose physical, chemical and other properties are known, and where it can be demonstrated that the use of the reference standard is toxicologically relevant.

To investigate the applicability of the RSs (benchmarking) approach in the validation and acceptance of *in vitro* tests, the ECVAM Reference Standards Working Group has been established, with the following membership: Michael Balls (ECVAM, Italy), Lesley Earl (Unilever Research, UK), Julia Fentem (ECVAM) and Richard Lewis (Zeneca CTL, UK).

Criteria for the selection, use and validation of reference standards

The ECVAM working group on reference standards agreed that the following criteria should be applied to determine whether a substance is a suitable RS for use in a given *in vitro* test:

1. The RS should be readily available in a chemically pure and stable form.
2. The RS should provide reproducible results within the test system of choice.
3. The RS should be associated with *in vivo* data (preferably human) of high quality and low variability.
4. The set of RSs chosen should cover the full range of the *in vivo* toxicological endpoint, which should be clearly defined.

Having established a set of suitable RSs, the following points should be considered before they are used in the testing of chemicals: a) the relative toxicity of the RS and test material (if known); b) the chemistry (including structure and functional class) and physical form of the RS relative to the test substance; and c) the likely mechanisms of toxicity (if known) of the RS and test substance.

The criteria for determining whether an assay and its associated reference standards are ready for prevalidation and validation are similar to the criteria applied to any alternative method:

1. The method and RSs must be well-developed and associated with good supporting data.
2. The method and RSs must be relevant to the toxicological endpoint.
3. There must be a protocol and prediction model covering the use of the RSs.
4. There must be evidence that the reproducibility of the RSs is adequate for the purpose.

An ECVAM study to evaluate the use of reference standards in the validation process

The ECVAM Reference Standards Working Group decided that an initial evaluation of the benchmarking approach should be made by concentrating on eye irritancy as the toxicological endpoint. There are a number of reasons for this decision: a) several validation studies for eye irritation have so far failed to find suitable alternatives, which are urgently required; b) several *in vitro* eye irritation assays are promising candidates for evaluation by the reference chemicals approach; c) there are a few groups of chemicals which could be considered as candidate RSs available in the public domain; d) good quality human exposure data could be available for some chemicals; and e) there is a large industrial community which has a wealth of experience in using reference standards for assessing eye irritancy.

The details of an ECVAM-sponsored study are currently being finalised. It is envisaged that five *in vitro* methods will be included in the study: a) the ICE test; b) the bovine corneal opacity/permeability (BCOP) test; c) the combined use of the HET-CAM and NRU tests; d) EpiOcular^{TM}; and e) the RBC haemolysis test. For each method, chemicals belonging to one or more chemical groups will be tested in a single laboratory. Most of the chemical groups will be defined in terms of functional class and/or physical form, although a mixed group will also be tested in each laboratory. The testing of chemicals will be carried out in two phases. In the first phase, each laboratory will be required to test up to five chemicals per chemical group (these will be the RSs) and to develop a PM. At this stage, the chemical identities and accompanying *in vivo* data will be supplied to the laboratories. In the second phase of testing, each laboratory will be required to repeat the testing of the RSs in phase I and to test a further five chemicals per chemical group, which will supplied coded. Each laboratory will be required to predict the eye irritation potential of the five test chemicals, using the PM developed in phase I. The reliability and relevance of each *in vitro* test, as judged by the benchmarking approach, will be assessed by independent data analysis.

Harmonisation of Classification Systems

One of the reasons why no *in vitro* test for eye irritation has yet been validated relates to the choice of statistical method in the validation studies. For example, in the EC/HO and COLIPA validation studies, the predictivities of the *in vitro* methods were assessed by correlating the *in vitro* scores with the MMAS. The problem with this approach, however, is that the MMAS is highly variable, which means that the *in vitro* methods are being judged against a variable for which there is a considerable degree of uncertainty. One way of avoiding the use of MMAS as the *in vivo* endpoint is to use classifications of eye irritancy instead, but this presents the difficulty of choosing an appropriate classification system. Since it is desirable that international validation studies adopt an internationally recognised classification system, a possible solution would be to use the proposed OECD system for the harmonised classification of eye irritants/corrosives (OECD, 1998). An evaluation of the proposed OECD system, carried out by Menk Prinsen (TNO, Zeist, The Netherlands), shows that the OECD classification system is broadly comparable to the current EU system (Appendix 1).

An important consideration in the development of any classification system, particularly the proposed OECD system, is the treatment of results obtained for solid materials. In the

Draize test, solids are instilled as a bulk material in the conjunctival sac, where they may be entrapped for up to 24 hours. If the substance is poorly soluble and has cytotoxic properties, the combined effect of mechanical damage and cytotoxicity can cause very severe effects. Such a high and persistent exposure does not occur in rabbits when testing liquids for eye irritation, nor does it occur when testing compounds for skin irritation (dermal exposure of 4 hours). It is a situation which represents a highly unusual case of accidental human exposure, and which cannot be mimicked by many alternatives. An example of the effects of solid entrapment is provided by sodium perborate (chemical #37 in the EC/HO study). The *in vivo* data for this substance (ECETOC, 1992 & 1998) show that the corneal opacity was slight or moderate 1h after treatment, and covered only a small part of the cornea (the lower part), whereas the conjunctival swelling was severe. It is not clear from the data whether solid remains were still present in the conjunctival sac at the 24h reading, or whether rinsing of the eye was performed. However, in two rabbits, the maximum corneal opacity (score of 4) was observed 21 days after treatment. As a result of this effect, sodium perborate is classified as R41/Category A (Appendix 1), even though it has a relatively low MMAS (score of 30) and caused low *in vitro* scores in several assays (Balls *et al.*, 1995). Similarly, four other solid materials (captan 90 concentrate, quinacrine, and 1-napthalene acetic acid) which caused low *in vitro* scores in the EC/HO study are also classified as R41/Category A (Appendix 1).

One of the disadvantages of testing solids in the standard rabbit eye test is the amount of test substance which is typically instilled into the rabbit eye. OECD Guideline 405 (OECD, 1987) recommends using either a volume of 0.1 ml of solid (in the form of a fine, but slightly compacted, dust) or a weight of no more than 0.1g. However, because the density of solids is often much higher than 1 kg/l, overdosage may occur, thereby contributing to the variability in the eye effects. The disadvantages of testing solids are discussed in more detail by Walker (1985), who contends that the use of a lower volume of test material, placed directly on the cornea, gives a much better prediction of eye irritation in humans.

Multivariate Statistical Analysis

The techniques of multivariate statistics are used to investigate complex data sets, i.e. data sets comprising many variables (often correlated with one another) for a given set of objects. In toxicological applications, the objects are generally chemicals or formulations, and the variables are generally physicochemical properties or biological endpoints.

Principal components analysis

Principal components analysis (PCA) is a method for reducing the number of variables in a complex data set with the minimum loss of information (variance). The original variables are transformed into new variables called principal components (PCs), which are linear combinations of the original variables. The PCs are constructed in such as way that: a) all PCs are orthogonal (uncorrelated); and b) the first PC accounts for the greatest proportion of the variance in the original data set, while subsequent PCs account for decreasing proportions of the remaining variance. The PCs can be interpreted in terms of their vector loadings, which are simply the coefficients in the linear combination of original variables: the greater the loading of a PC for a particular variable, the more the PC is composed of that variable.

An example of the use of PCA is provided by Barratt *et al.* (1998). PCA was used to visualise the relationship between the skin corrosivity potential of four groups of chemicals

(acids, bases, electrophiles and neutral organics) and their physicochemical properties. The PCA plot for each group of chemicals showed a general separation between the corrosive chemicals and the non-corrosive chemicals, and enabled borderline chemicals to be identified. This information was used in the ECVAM validation study on alternatives to the skin corrosivity test, to guide the selection of test chemicals, and to assist the interpretation of *in vitro* data .

Another example of the application of PCA is provided by Lovell (1996a & 1996b). PCA was used to obtain the PCs of 18 rabbit eye tissue scores (referring to damage of the cornea, conjunctiva and iris at three time points). The first PC, which accounted for 77% of the variability in the *in vivo* data, gave approximately equal weight to the 18 tissue scores and was strongly correlated with the MMAS, whereas the second PC, which accounted for 7% of the variability, was found to contrast damage to the cornea and iris from damage to the conjunctiva. It was concluded that there is only limited evidence for differential responses of the different tissues (within 24-72h of treatment), and that alternative methods which are developed to reproduce the MMAS are unlikely to be predictive of individual tissue scores.

Partial least squares

Partial least squares (PLS) analysis is similar to PCA in that it reduces the number of variables in a complex data set, but it differs in that the variables are divided into two subsets, relating to the dependent variables and the independent variables. PCA is carried out on each subset of variables, and multiple regression is used to correlate the PCs of the dependent variables with the PCs of the independent variables. The PCA and multiple regression are carried out so as to preserve as much of the variance in the dependent and independent variables as possible, while at the same time maximising the strength of the correlation between the dependent variables and the independent ones. The results of PLS analysis are visualised as projections on two-dimensional maps. Variables which project furthest from the origin of the graph are the most relevant, whereas those located closest to the origin are the least relevant. Variables which project close to one another are positively correlated, whereas those which project diametrically opposite to one another are negatively correlated. Further details of the PLS method are provided by Lindberg *et al.* (1983).

A study carried out by de Silva *et al.* (1997) illustrates the use of PLS in establishing batteries of *in vitro* alternatives to the eye irritation test. The technique was applied to a data set consisting of 11 *in vitro* endpoints (relating to eight tests) and 27 *in vivo* endpoints for a set of 32 surfactants and surfactant-based formulations. The analyses indicated that the most predictive methods were the HET-CAM testr, the BCOP assay, and the NRU-SIRC assay.

Cluster analysis

Cluster analysis (CA) is a method for visualising (and quantifying) the similarity between different objects (chemicals), or between different variables (physicochemical and toxicological endpoints). The objects or variables are placed in multi-dimensional space so that adjacent observations can be grouped in a stepwise fashion. This results in a dendrogram in which all of the observations are grouped into one or more clusters. The similarity between observations is defined as the distance (typically, the Euclidean distance) between them. There are various types of clustering algorithm, which can be distinguished according to: a) the number of links they allow between observations; b) whether they allow links to be broken

once they have been formed; and c) whether clusters are built up from individual observations, or split off from a single cluster containing all observations. Further details on cluster analysis are given by Gordon (1981).

A possible use of CA would be the clustering of *in vitro* endpoints to help in selection of tests for inclusion in a testing strategy. To illustrate this application, 16 *in vitro* endpoints for predicting eye irritation potential were clustered on the basis of the *in vitro* scores for 43 chemicals (Figure 3). It can be seen that the cell-based assays cluster together, as do the organotypic assays, even though the distinction between the two types of assays was not fed into the clustering process. If CA were used in the design of a testing strategy, tests would be chosen from different clusters since this would maximise the amount of information provided by the strategy as a whole, i.e. the tests would be selected on the basis of their dissimilarity.

Linear discriminant analysis

Linear discriminant analysis (LDA) is a method for classifying objects into two or more groups on the basis of one or more variables. It works by 'plotting' the objects in one-dimensional or multi-dimensional space (depending on whether one or more variables are being used), and by constructing one (or more) linear boundaries which separate the objects into two (or more) groups. Each boundary is defined by a linear equation which contains as many terms as there are variables, and which is used in the classification of objects. In the simplest applications of LDA, there is a just a single boundary between two groups. If only one variable is used, a point-like boundary results, which can be used as a cut-off value for classifying the objects into the two groups. If two variables are used, the boundary can be thought of as a plane, and if three or more variables are used, the boundary becomes a hyperplane in multi-dimensional space. In situations where there are many potentially useful variables, stepwise LDA can be used to choose the variables which provide the best discrimination between groups. An introduction to LDA is given by McFarland & Gans (1990).

LDA can be used to derive PMs for predicting the toxicological classifications of chemicals. An example is provided by Spielmann *et al.* (1996), who report that the combined use of HET-CAM and the 3T3 NRU test provides a satisfactory means of distinguishing severely irritant (R41) chemicals from non-severely irritant chemicals. Stepwise LDA of ten endpoints (nine HET-CAM and one 3T3 NRU) showed that the best discrimination was achieved by using a single HET-CAM endpoint (the time taken for coagulation to occur) rather than the usual weighted combination of endpoints based on haemorrhage, lysis and coagulation. The addition of the 3T3 NRU endpoint to the model improved the identification of R41 chemicals which cause irreversible effects (according to a recent modification of the EU guideline [EEC, 1992], any chemical which causes an irreversible eye effect is classified as R41, regardless of the degree of that effect). On the basis of the LDA analyses, various testing strategies based on the sequential application of HET-CAM and the 3T3 NRU were proposed. Depending on the solubilities of the test substance in oil and water, slightly different PMs were recommended for converting *the in vitro* test results into predictions of eye irritancy.

Testing Strategies

The use of stepwise (hierarchical) testing strategies, which incorporate a range of alternative methods (structure-activity relationships, biokinetic models, physicochemical techniques, and

in vitro tests) and which use animals only if necessary, is increasingly being considered as the most effective way of predicting toxicity while at the same time minimising animal testing. In addition to providing a means of implementing the Three Rs, testing strategies optimise the use of existing knowledge and resources, and promise to improve the scientific basis of toxicity testing. This is particularly true in the case of eye irritancy testing, since it seems unlikely that any single *in vitro/ex vivo* test will be capable of reproducing the complexity of the *in vivo* response.

Hierarchical testing schemes have been proposed in the literature for a variety of toxicological endpoints, including skin irritation/corrosion (Basketter *et al.*, 1994), skin sensitisation (Basketter *et al.*, 1995), phototoxicity (Spielmann *et al.*, 1994) and neurotoxicity (Atterwill *et al.*, 1994). For the assessment of eye irritancy, there have been several proposals based on the combined use of a cytotoxicity test and an organotypic test; examples include: a) the 3T3 NRU cytotoxicity and HET-CAM tests (Spielmann *et al.*, 1996); b) the K562 cytotoxicity and isolated rabbit eye (IRE) tests (Lewis *et al.*, 1994; Purchase *et al.*, 1995); and c) the 3T3 NRU cytotoxicity and IRE tests (D.J. Esdaile, personal communication). The proposal made by Spielmann *et al.* (1996) for the combined use of 3T3 NRU and HET-CAM consists of a tiered strategy for the identification of severe eye irritants (R41 chemicals), as defined by EU criteria (EC 1993). The strategy can be applied to chemicals with different solubility characteristics, since separate PMs were derived using the data generated in the German validation study on alternatives to the Draize test (Spielmann *et al.*, 1996).

At the regulatory level, a tiered approach to eye irritancy/corrosivity testing is provided for in the 1987 update of OECD Guideline 405 (acute eye irritation/corrosion; OECD, 1987), although no particular testing strategy is specified. A proposal for a testing strategy (Figure 1) was discussed at an OECD workshop on *Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods*, held in Solna, Sweden, in January 1996. Subsequently, the proposed strategy was modified by the OECD Advisory Group on Harmonization of Classification and Labelling, who incorporated a *Testing and Evaluation Strategy for Eye Irritation/Corrosion* (Figure 2) into their revised proposal for the harmonization of hazard classification based on eye irritation/corrosion (OECD, 1998). Important features of the proposed OECD strategy are: a) it allows for the classification of chemicals as irritant or corrosive to the eye on the basis of validated alternative methods; b) it only permits the use of animal tests to check negative results (non-irritant and non-corrosive) generated by one or more alternative methods; and c) animal testing is minimised by using a single rabbit test to detect serious damage to the eyes (in which case no further testing would be conducted) before conducting one or two additional rabbit tests to detect moderate irritancy. An assessment of this testing strategy has been carried out by ECVAM (Appendix 2). The outcome of this assessment is intended to illustrate a general approach to the evaluation of stepwise testing strategies, which has also been applied to the proposed OECD testing strategy for skin corrosion (Worth *et al.*, 1998).

Conclusions and Recommendations

General

1. One of the reasons that several promising *in vitro* tests for eye irritation have been unsuccessful in previous validation studies is that the tests were judged on their ability to predict the MMAS. In addition to being a highly variable measure of *in vivo* irritancy, the MMAS is of questionable relevance to the human eye since it is defined in terms of an arbitrary weighted combination of different tissue scores (relating to effects in the cornea, conjunctiva and iris). It is more realistic to evaluate *in vitro* tests in terms of their ability to predict individual tissue scores, or in terms of their ability to classify chemicals. In the latter case, a suitable classification scheme would be the OECD proposal for the harmonised classification and labelling of chemicals based on eye irritation/corrosion.

2. When assessing the predictivity of an alternative method during a validation study, a useful consideration is the best possible predictivity which can be expected on theoretical grounds. This can be estimated by carrying out computer simulations which model the effect of variability in the *in vivo* data on the strength of the *in vitro-in vivo* relationship.

3. The use of reference standards is widespread in industry, where they are used with great effect to make safety decisions. It is highly desirable that the knowledge which exists in industry is transferred to the regulatory bodies responsible for the hazard and risk assessment of new chemicals and formulations. It is important that agreement is reached on the chemicals which can be used as reference standards. These chemicals should be well-characterised, readily available, and associated with high quality *in vivo* data (preferably obtained in accordance with international test guidelines).

4. The use of reference standards could provide a new way of validating *in vitro* tests, particularly in cases where quantitative comparisons between *in vitro* and *in vivo* data are confounded by the high variability of the *in vivo* data. Therefore, ECVAM intends to carry out a pilot study to evaluate the usefulness of reference chemicals in the validation of alternatives to the Draize eye irritation test.

5. The development and implementation, in a regulatory testing framework, of appropriate testing strategies for eye irritation, which limit the use of the Draize rabbit test to the final step, are critically dependent on the availability of one or more scientifically validated *in vitro* tests for inclusion in the testing strategy. Therefore, ways must be found to either demonstrate that the *in vitro* tests currently being used in house are indeed valid for the purposes to which they are being put, or new *in vitro* tests will need to be developed and validated.

6. Test batteries combining a cytotoxicity test with an organotypic test appear to provide an effective means of identifying irritant chemicals, at least for screening purposes. The combined use of the 3T3 NRU and HET-CAM tests has been shown to be highly predictive of severe eye irritants in a validation study carried out in Germany.

Recommendations to the regulatory community

7. OECD Test Guideline 405 (acute eye irritation/corrosion) should be modified with respect to a) the *in vivo* testing of solid materials - the solid material should be removed from the eye after treatment, to produce a more relevant and reproducible exposure, and to reduce unnecessary animal suffering; b) the use of pH measurements - one or more PMs for converting pH measurements into predicted classifications of eye irritancy should be cited, along with the chemical concentration at which the pH measurements should be carried out.

8. The recommendation that measurements of acid/alkali reserve should be carried in addition to pH, made in OECD Test Guideline 405 and in the proposed OECD testing strategy for eye irritation/corrosion, should be reconsidered, since it is questionable that acid/alkali reserve yields any more information than is provided by pH alone.

9. The proposed OECD testing strategy for eye irritation/corrosion should incorporate one or more *in vitro* methods in Step 5a (screening of severe irritants). Suitable candidates would be the HET-CAM and 3T3 NRU tests, which have been validated in a study carried out in Germany.

Recommendations for further research

10. The predictive abilities of several testing strategies for eye irritation should be evaluated. The evaluations should include an assessment of the validation status of the component tests and of the testing strategies as a whole.

11. Further research is needed on the QSAR modelling of eye irritancy, including the development and evaluation of models for predicting levels of irritancy (e.g. R41/R36/NI).

12. There is a need for alternative methods which are capable of modelling the persistence or reversibility of eye effects. For example, an *in vitro* assay for reversibility could be based on the release of inflammatory mediators.

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光毒性　被験物質の一覧

Chemical	in vitro（下段は文献）									in vivo	
	3T3 NRU	Skin2TM	真菌	赤血球溶血	Hb光酸化	His光酸化	光binding*	補体活性化法	OLATEX P	human	animal
	A,B,C,D	A,D	A,F,G	A,H,I	H	A,J	A,K	A,L	A	A	A
Phase I											
class I:UVA-absorbing, phototoxic											
Promethazine	+	+		+	+				+	+	+/−
Chlorpromazine	+	+	+	+	+		+		+	++	++
6-Methylcoumarin	+	−	+	+//−	+	+	+		+	a	a
Tetrachlorsalicylanilide(TCSA)	+	+	+	+	+	+	+		+	a	+
Doxycycline	+	+	−	−	+	+			+	+	+
8-Methoxypsoralen	+	+	+	−	+	−			−	++	++
Tetracycline	+	+	−	−	+	−			+	+	+
Amiodarone	+	+	−	+	+				+	+	+
Bithionol	+	−	−	+	+		+		+/−	+	+
Neutral red	+	+		+	+				+	+	
Rose bengal	+	+		+	+	+			+	+/−	−
class II:UVA-absorbing, non-phototoxic											
(Piroxicam)	−	−	−	−	−	−			−	(+)	−
Cinnamic aldehyde	−	−	+	−	+	−			−	a	a
Chlorhexidine	−	−		+	−				−		
Uvinul MS40	−	−		−	−				+	+/−	
p-Aminobenzoic acid(PABA)	−	−		−	−				−	a	
class III:non-UVA-absorbing, non-phototoxic											
Penicillin G	−	−		−	−				−		
L-Histidine	−	−		−	−				−		
Thiourea	−	−		−	−					a	
Lauryl sulfate	−	−		−	−				−		
Phase II											
2-Hydroxy-4-methoxybenzophenone	−		−							+/−	
5-Methoxypsoralene(5-MOP)	+		+	−						+	+
Acridine-hydrochloride	+		+	+		+				+	+
Acridine-free base	+									+	+
Amiodarone	+		−			−				+	+
Anthracene	+		+	+		+				+	+
Bergamot oil	+									+	+
Bithionol	+									+	S+/T−
Chlorhexidine dihydrochloride	−									−	
Chlorpromazine	+		+							+	+
Demeclocycline	+									+	+
Fenofibrate	+									(+)	
Furosemide										+	
Hexachlorophene	−(UVB+)									(+/−)	−
Ketoprofen	+									(+/−)	−
Sodium lauryl sulfate	−										
Musk ambrette	+		−//+	+			+			(+/−)	−
Nalidixic acid-sodium salt	+		−			+				+	+
Nalidixic acid-free acid	+									+	+
Neutral red	+									+	+
Norfloxacin	+									+	+
Ofloxacin	+									+	+
p-Aminobenzoic acid(PAVA)	−									(+/−)	−
Penicillin G sodium salt	−										
Promethazine	+						+			+	+
Protoporphyrin IX-free acid	+										+
Protoporphyrin IX-disodium salt	+										+
Rose bengal	+		+							+	−
Tiaprofenic acid	+									+	+
COLIPA最終追加											
Octyl salicylate	−									−	
Octyl methoxycinnamate	−									−	
Benzylidene camphor sulphonic acid and salts	−									−	
4-Methylbenzylidene camphor	−									−	
3-Benzylidene camphor	−									−	
Terephthalidene dicamphor sulphonic acid and salts	+									−	
Polyacrylamidomethyl benzylidene camphor	−									−	

*:ヒト血清アルブミンとの結合
// で区切った部分は両方のデータあり
保体活性化法は原著を取り寄せ中

a: photoallergen

光毒性　被験物質の一覧

Chemical	in vitro(下段は文献)									in vivo	
	3T3 NRU	Skin2TM	真菌	赤血球溶血	Hb光酸化	His光酸化	光binding*	補体活性化法	BOLATEX P	human	animal
	A,B,C,D	A,D	A,F,G	A,H,I	H	A,J	A,K	A,L	A		G,I
Phasel の検討(追加)											
Plant extract											
Umbelliferae			+								
Giant hogweed			+								
Cow-parsnip			+								
chrysanthemum, tansy, yarrow, sagebrush, ragweed			−			+					
wild feverfew, cornflower, coreopsis			−								
aster, burweed			−								
dahlia, lettuce, chicory, glden-rod			−			+					
black-eyes Susan, burdock, sunflower, tocklebur			−								
dog fennel, sneezeweed			−			+					
Chemicals											
Alantolactone			−								
Isoalantolactone			−								
Trimethylpsoralen			+								
kinurenic acid, potassium dichromate						+					
Fragrance materials											
Oil of bergamot			+								
custus-root-oil			+			−					
cinnamyl alcohol			+			−					
amylcinnamaldehyde			+			+					
jasmine mix			+			+					
laurel leaf oil			+			−					
colophony			+			+					
oak moss			−			+					
balsam of Peru			−								
usnic acid			−								
isoeugenol, hydroxycitronella, eugenol			−			−					
geraniol, benzylbenzoate, benzylalcohol			−			−					
methylsalicylate			−			−					
benzylbenzoate						−					
Dyestuffs											
Benzanthrone			+								
eosin, toluidine blue			+								
methylene blue			+			+					
Anthraquinone			−								
Drugs											
benoxaprofen			+			+					
azapropazone, carbamazepine, cimetidine,			−								
dimethylchlorotetracycline			−								
diflusinal, oxytetracycline, propranolol,			−			+					
griseofulvin, imipramine, methyl-DOPA,			−			−					
hydrochlorthiazide			−								
protriptyline			−			+					
trimethoprim, sulphamethoxazole, sulphapyridine			−			−					
thinylestradiol, minocycline						−					
その他(追加)											
Sulfanilamide			−	−			+				−
Buclosamide							+				
Fenticlor							+				
Omadine(sodium salt)							+				
Quinoxaline-1,4-dioxide							+				
Triacetyldiphenolsatine							+				
Tribromosalicylanilide			−	+			+				
Musk Ketone			−	−							−
Musk Xylene			−	−							−
Phantolide			−	+							+
Galaxolide			+	+							+
Parsol-1789			−	−							−
Parsol MCX			−	−							−
ASL-24S			−	−							−
Escalol 507			+	−							+
Indomethacin			−	−							−//a
TCC			−	−							−

分担研究報告書

In vitro 経皮吸収試験法ガイドラインについて
安息香酸ナトリウム、サリチル酸経皮吸収試験

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要約

１９９６年６月、OECD は、In vitro 経皮吸収試験法の第２次ガイドライン（案）を作成し、各国に公表した。我が国では、国立医薬品食品衛生研究所の大野薬理部長を中心として意見の取りまとめ、同年１０月に OECD に意見を提出した。この第２次 OECD ガイドライン（案）に対する回答が OECD から提出されていないが、予め、「In vitro 試験法を用いた化粧品の安全性評価法およびその国際的ハーモナゼーションによる研究」の一環として、In vitro 経皮吸収試験法を防腐剤の一種である安息香酸ナトリウムおよびサリチル酸の経皮吸収試験に実施した。縦型の Franz 型拡散セルを用いて検討し、それら試験物質の donor 側の vehicle への溶解性を考慮することが、In vitro 経皮吸収試験を実施する場合の大きな要因であることが示唆された。

キーワード：OECD ガイドライン、In vitro 経皮吸収試験、
安息香酸ナトリウム、サリチル酸

A. 研究目的

医薬品、化粧品あるいは添加物等の in vitro 経皮吸収試験法は、適切な実験原理に基づき、適切な解析を行い、in vitro 経皮吸収試験法の結果を正確に記述できるデータを提示する必要がある。１９９６年６月、OECD は、the European Cosmetic Toiletry and Perfumery Association および European Center for the Validation of Alternative Methods のガイドラインを基に第２次 OECD ガイドライン（案）を作成し、各国に公表した。このガイドライン（案）に関して、我が国では、国立医薬品食品衛生研究所の大野薬理部長を中心として意見の取りまとめ

を行い、同年１０月に意見を OECD に提出したところである。この第２次 OECD ガイドライン（案）に対する回答が OECD から報告されていないが、予め、「in vitro 試験法を用いた化粧品の安全性評価法およびその国際的ハーモナゼーションによる研究」の一環として防腐剤の一種である安息香酸ナトリウム（ＢＡ）およびサリチル酸（ＳＡ）の経皮吸収的な安全性を評価するため、in vitro 経皮吸収試験を縦型の Franz 型拡散セルを用いて検討した。また、それら化合物を化粧水および乳液に添加したときの in vitro 経皮吸収試験を実施し、vehicle の経皮吸収試験に及ぼす影響を検討したので報告する。

B．研究方法

１）試薬および試液

ＢＡ、ＳＡ、液体クロマトグラフ（ＨＰＬＣ）用アセトニトリルおよびＨＰＬＣ用メタノールは和光純薬工業株式会社より購入した。ＨＰＬＣ用カラムは CAPCELL PAK C_{18} カラム（粒径５μm、内径 4.6mm，長さ 25cm）を資生堂から購入した。化粧水および乳液は市販品を用いた。その他の試薬は，試薬特級品を用いた。

ＢＡ原液：ＢＡ約０．１ｇを精密に量り，１／３０Ｍリン酸緩衝液（ｐＨ７．４）に溶かし、正確に１０ｍＬとした（１ W/V%）。

ＢＡ含有化粧水：ＢＡ約０．１ｇを精密に量り、化粧水１０．０ｍＬに溶解した。

ＢＡ含有乳液：ＢＡ約０．１ｇを精密に量り、乳液１０．０ｇに溶解した。

ＳＡ原液：ＳＡ約０．０２ｇを精密に量り，生理食塩液に溶かし，正確に１０ｍＬとした（0.2 W/V%）。

ＳＡ含有化粧水：ＳＡ約０．０２ｇ精密に量り、化粧水１０．０ｍＬに溶解した。

ＳＡ含有乳液：ＳＡ約０．０２ｇ精密に量り、乳液１０．０ｇに溶解した。

２）剥離皮膚の調製法

モルモット（ハートレー系、オス、２５０－３００ｇ）をエーテル麻酔後，エーテルでの深麻酔および呼吸停止により処理した。腹部の毛を電気バリカンで除いた後，腹部の皮膚を剥離した。皮下脂肪を除き，凍結保存し，必要なときに解凍して用いた。

３）ＢＡおよびＳＡの皮膚透過実験

モルモット剥離皮膚を縦型の Franz 型拡散セル（static セル系、有効透過面積 0.246cm^2）に表皮を donor 側に向けて装着した。donor 側にＢＡあるいはＳＡ原液１．０ｍＬを加え、receptor 側に１／３０Ｍリン酸緩衝液（ｐＨ７．４）（ＢＡの場合）あるいは生理食塩液 19ml（ＳＡの場合）を加え、３２℃で２～２４時間後（ＢＡの場合）あるいは２～８時間に側の溶液 0.2ml を分取した。この液２０μＬを用いて HPLC 法にて測定を行い，あらかじめ作成した検量線を用いて透過したＢＡあるいはＳＡ量を求めた。なお，側に生理食塩液 0.2mＬを加え，receptor 側の容量を一定とした。

（HPLC 条件）

検出器：紫外吸光光度計（測定波長：230nm）

カラム：資生堂製 CAPCELL PAK C_{18} カラムを用いた。

カラム温度：４０℃付近の一定温度

移動相：５０ mM リン酸塩緩衝液（ｐＨ２．５）／アセトニトリル混液（７：３）（ＢＡの場合）あるいは５０ mM リン酸塩緩衝液（ｐＨ４．０）／アセトニトリル混液（１７：３）（ＳＡの場合）

流量：１ｍＬ／ｍｉｎ

C．研究結果

１）ＢＡおよびＳＡの検量線および再現性

ＢＡ０．２～１．２μｇ／ｍＬおよびＳＡ０．２５～２．５μｇ／ｍＬの溶液２０μＬを用いてHPLC 条件にて検量線の作成を行った。その結果をFig.1 に示した。濃度と Peak area の間には原点を通る良好な直線関係を示した。また、ＢＡ０．２μｇ／ｍＬおよび１．０μｇ／ｍＬの２０μＬを用いたときの再現性は、それぞれ、１４２５６μＶ×sec（相対標準偏差１．７２％）と６８３５０μＶ× sec（相対標準偏差１．０６％）であった。ＳＡ０．２５μｇ／ｍＬおよび２μｇ／ｍＬの場合、それぞれ、１０９９５μＶ×sec（相対標準偏差３．２７％）と９１８４６μＶ×sec（相対標準偏差０．２９％）であった。

２）ＢＡの剥離皮膚透過について

ＢＡ原液（１０ｍｇ／ｍＬ）およびＢＡ含有化粧水１．０ｍＬ並びにＢＡ含有乳液１．０ｇを縦型の Franz 型拡散セルの donor 側に入れ、２～２４時間後に側に透過するＢＡ量を測定した。その結果を Fig.2 に示す。ＢＡを１／３０Ｍリン酸緩衝液（ｐＨ７．４）に溶かして donor 側に添加した場合に比べて化粧水あるいは乳液に溶かした場合の方がより早くモルモットの剥離皮膚を透過することが分かった。Table 1 に実験回数３～４回の透過速度の平均値および lag time を示した。また、Fig.3a にＢＡ含有化粧水を用いたときの８時間後のＨＰＬＣクロマトグラムを示した。ＢＡの保持時間は７．４分であった。

３）ＳＡの剥離皮膚透過について

ＳＡ原液およびＳＡ含有化粧水１．０ｍＬ並びにＳＡ含有乳液１．０ｇを縦型の Franz 型拡散セルの donor 側に入れ、２～８時間後に側に透過するＳＡ量を測定した。その結果を Fig.3 に示した。ＳＡの皮膚透過は、乳液、化粧水および生理食塩液の順序であった。Table 1 に実験回数４～５回の透過速度の平均値および lag time を示した。また、Fig.3b にＳＡ含有化粧水を用いたときの８時間後のＨＰＬＣクロマトグラムを示した。ＳＡの保持時間は６．３分であった。

D．考察

今回の実験条件を OECD ガイドライン（案）に基づいて Table 2 に示した。

3.Skin membrane の部分がＯＥＣＤガイドライン（案）では、ダーマトームで厚さ（２００－４００μｍ）に調製した split thickness skin を用い、原則的に、full thickness skin を実験に用いないとなっているが、今回は、full thickness skin を実験に用いた。4.Skin integrity は、OECD ガイドライン（案）では、浸透特性が既知の標準物質（例えば、トリチウム水）の浸透性を測定して調べることができるとなっているが、放射性同位体を用いる実験であるため、実施しなかった。また、5.Skin metabolism は、モルモットの腹部剥離皮膚を使用前まで凍結保存し、使用時に解凍して用いたため、皮膚代謝の実験を行っていない。11.Terminal procedures は、実験終了後、皮膚中に残存する物質を測定する必要があり、今回の実験では実施しなかった。

これらの OECD ガイドライン（案）に記載された何項目かを満たさない方法での実験であるが、今回実施した in vitro 経皮吸収実験で一応の傾向を掴むことができた。即ち、donor 側に１／３０Ｍリン酸緩衝液（ｐＨ７．４）に溶解した１０ｍｇ

／ｍＬのＢＡ溶液１ｍＬを用いた実験では、Fig.2から分かるように時間の経過と共に receptor 側に透過するＢＡの量は直線的に増加し、直線の勾配から得られて Flux は 4.70 μｇ／ｃｍ²／ｈｒ（Table 1）であったが、vehicle を化粧水あるいは乳液に交換すると Flux は 10.22, 8.69 μｇ／ｃｍ²／ｈｒに増加した。また、donor 側に生理食塩液に溶解した２ｍｇ／ｍＬのＢＡ溶液１ｍＬを用いた実験では、Fig.3 から分かるように時間の経過と共に receptor 側に透過するＳＡの量は直線的に増加し、直線の勾配から得られて Flux は 44.94 μｇ／ｃｍ²／ｈｒ（Table 1）であったが、vehicle を化粧水あるいは乳液に交換すると Flux は 20.89, 3.46 μｇ／ｃｍ²／ｈｒに減少した。この違いは、安息香酸ナトリウムあるいはＳＡの vehicle への溶解性の違いを反映していることが示唆された。n-octanol/vehicle へのＢＡあるいはＳＡの分配を調べることにより、その関連性を明らかにできることが期待される。

Lag time については、ＢＡの場合、vehicle が緩衝液と化粧水では同じ値（1.9 時間）を与え、乳液の場合と若干異なっていた。ＳＡの場合、各 vehicle により、Lag time が異なっていた。

今回の検討結果より、試験物質の donor 側の vehicle への溶解性を考慮することが、in vitro 経皮吸収試験を実施する場合の大きな要因であることが示唆された。