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SKIN2 AN IN VITRO HUMAN SKIN MODEL : THE CORRELATION BETWEEN IN VIVO AND IN VITRO TESTING OF SURFACTANT;DEMETRULIAS J.,ET AL
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CHARACTERIZATION AND APPLICATION OF HUMAN EPIDERMIS RECONSTRUCTED IN VITRO ON DE-EPIDERMIZED DERMA;PARNIGOTTO P.P.,ET AL
FARMACO,(1998),53,(2),125-31

USE OF DERMAL EQUIVALENT AND SKIN EQUIVALENT MODELS FOR IN VITRO CUTANEOUS IRRITATION TESTING OF COSMETIC PRODUCTS : COMPARISON WITH IN VITRO HUMAN DATA ;AUGUSTIN C.ET AL
J.TOXICOL.-CUT.& OCULAR TOXICOL.,(1998),17,5-17

THE ECVAM INTERNATIONAL VALIDATION STUDY ON IN VITRO TESTS FOR SKIN CORROSIVITY.
2.RESULT AND EVALUATION BY THE MANAGEMENT TEAM ;J.H.FENTEM
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THE ECVAM INTERNATIONAL VALIDATION STUDY ON IN VITRO TESTS FOR SKIN CORROSIVITY.
1.SELECTION AND DISTRIBUTION OF THE TESTS CHEMICALS.;BARRATT MD ET AL
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SKIN CULTURE MODEL:A POSSIBLE ALTERNATIVE TO THE USE OF EXCITED HUMAN SKIN FOR ASSESSING IN VITRO PERCUTANEOUS ABSORPTION ;DOUCET O., ET AL
TOXICOLOGY IN VITRO,(1998),12,295-304

THE INTERLABORATORY STUDY OF THE REPRODUCIBILITY AND RELEVANCE OF EPISKIN, A RECONSTRUCTED HUMAN EPIDERMIS IN THE ASSESSMENT OF COSMETICS IRRITANCY;ROGUET R.,ET AL
TOXICOLOGY IN VITRO,(1998),12,295-304

5 経皮吸収試験法検討の現状

「経皮吸収」とは、皮膚に適用された化学物質が、皮膚の表面の角質層あるいは毛のうから表皮を経て、真皮を通り、血液循環系の中に入っていくことである。化粧品あるいはそれらに使用される原料は、本来、皮膚にはもちろんのこと、人体に対する作用が緩和なものと規定されているが、それらが経皮吸収されて、どのような作用を及ぼすのかという知見を得ることは、刺激性や感作性、毒性の機序を理解し、安全性を評価、予測する上で、重要な情報を与えるものである。

一口に経皮吸収試験といっても、生体の皮膚に直接被験物質(例えば、放射線ラベルされた化合物)を適用し、体内への移行を測定する本格的な経皮吸収試験(in vivo 試験)の他に、実験動物から摘出、剥離した皮膚(ヘアレスマウス、ラット、モルモット、ユカタンミニブタや場合によっては、術後や死体からのヒト皮膚)を用いる in vitro 試験など、様々な方法が検討されており、後者は、簡便、迅速であるという理由だけでなく、動物愛護の点からも支持されている。しかし、これらを in vitro 試験と呼ぶかどうかは、論議のあるところで、1997 年 10 月の、OECD試験法のガイドライン作成のための、拡大推進委員会の中でも、"ex vivo 試験"と呼ぶべきである、とする考え方も出されている。さらに、動物実験代替法学会の支援を受けて、摘出皮膚の代わりに人工膜や、人工皮膚を用いた検討も進められている。

ここでは、主に最近の日本国内での報告例を挙げて、化粧品を取り巻く経皮吸収試験の現況について簡単に報告する。特に、医薬品の研究開発における、薬物の経皮吸収試験や、皮膚透過促進剤の開発のための研究報告は多く、その中には、化粧品の原料、あるいはそれに類似した物質について検討しているものもいくつかは見受けられる。しかし、化粧品に実際に用いられている原料、基材あるいは薬効成分などに関するもの、さらにそれらの安全性評価にまで言及した報告は少ない。また、実験動物からの摘出皮膚を用いない研究報告もほとんど見られないのが現状である。

以下に、いくつかの研究報告例を具体的に挙げてみる。

城西大薬学部の森本教授のグループは、ヘアレスラットの腹部摘出皮膚を用いて、化粧品にも使用される乳酸-エタノール-ミリスチン酸イソプロピル系が、フマル酸ケトチフェンの皮膚透過性を促進することを確認しており、また、本報では、Silicone 膜や Microporous 膜のような人工膜でも検討している。

・中村裕行(城西大・薬)ら:乳酸-エタノール-ミリスチン酸イソプロピル系の経皮吸収促進効果と機構に関する研究, 薬物動態, 8, 695-698, (1993)

青柳らは、経皮吸収促進効果がありながら、高分子

量であるために刺激性を低減させた、ポリエチレングリコール(PEG)/ポリジメチルシロキサン(PDMS)ブロックコポリマーがアンチピリンの経皮吸収促進効果があることを確認している。
・青柳隆夫(東京女子医科大):経皮吸収促進剤の皮膚に対する安全性の研究, フレグランスジャーナル, 1996-4, 34-41, (1996)

竹内らは、オレイン酸に類似したC18 で、二重結合の位置が異なるペトロセリン酸の皮膚透過促進のメカニズム解明のために、ラットの腹部皮膚を用いて、角質層や真皮の構造変化を FT-IR/ATR 法によって評価している。
・竹内由和(神戸学院・薬)、鈴木正夫(日本油脂(株))ら:ペトロセリン酸の皮膚透過促進メカニズム, 薬剤学, 57 Suppl., 228-229, (1997)

京都大学薬学部、橋田教授のグループは、モルモットやラットの胴部の摘出皮膚を用い、 d-リモネンやオレイン酸の吸収促進効果の解析や、マンニトールやブチルパラベンを含む各種薬物の透過のメカニズムを解明するとともに、さらに、新たに開発した吸収促進剤GACH(geranylazacycloheptan-2-one)の評価を行っている。
・ Yamashita F(Kyoto Univ.・ Pharm.)et.al.: In Vivo and in Vitro Analysis of Skin Penetration Enhancement Based on a Two-Layer Diffusion Model with Polar and Non-polar Routes in the Stratum Corneum, Pharm. Research, Vol11, No.2, 185-191, (1994)
・ Bando H(Kyoto Univ.・ Pharm.)et.al.: Analysis of in vitro skin penetration of acyclovir prodrugs based on a diffusion model with a metabolic process, Int.J.Pharm., Vol135, 91-102, (1996)
・ Bando H(Kyoto Univ.・ Pharm.)et.al.: Effects of Skin Metabolism on Percutaneous Penetration of Lipophilic Drugs, J.Pharm.Scie., Vol86, No.6, 759-761, (1997)

ラットやモルモット以外の実験動物を利用した例としては、昭和薬大の藤井らが、皮膚の構造がよりヒトに近いという理由で、ユカタンミニブタの皮膚を用い、Ibuprofen、Indomethacin、Antipyrine 等の各種薬物の皮膚透過性や、メントキシプロパンジオールとメントールによるインドメタシンの皮膚透過促進作用を検討している。
・藤井まき子(昭和薬科大)ら:メントキシプロパンジオールとメントールによるインドメタシンの Yukatan Minipig 皮膚透過促進作用, Drug Delivery System, Vol12, No.2, 127-131, (1997)
・ Fujii M (Showa College of Pharm.Sci.) et.al. : Evaluation of Yucatan Minipig Skin for Use as an in Vitro Model for Skin Permeation Study , Biol.Pharm.Bull., Vol20, No.3, 249-254, (1997)

化粧品原料の経皮吸収に関する研究としては、資生堂の熊野らが、新規の美白有効成分、アスコルビン酸-2-グルコシドの有効性をB16 メラノーマ細胞を使用して確認後、日本人男性5名により吸収試験を実施している。
・ Kumano Y (Shiseido) et.al. : In vitro and in vivo prolonged biological activities of novel vitamin C derivative,2-O-alpha-D-glucopyranosyl-L-ascorbic acid (AA-2G) ,in cosmetic fields , J.Nutr.Sci.Vitaminol., 44, No.3, 345-359, (1998)

順天堂大の相川らは、ヘアレスマウスの背部皮膚とヒト(腹部手術時の余剰皮膚)を使用して、グルコースやポリエチレングリコールの角層透過性を調べるとともに、アトピー性皮膚炎患者から採取した角層シートを用いて、健常人の透過性と比較し、皮疹部無疹部ともに透過性が亢進していることを確認している。
・相川洋介(順天堂大・医)ら:diffusion chamber を用いた正常ヒトおよびアトピー性皮膚炎患者における角質透過性の検討, 日皮会誌, 107(12), 1473-1478, (1997)

化粧品の安全性に関わるものとしては、パラベン類やサリチル酸の経皮吸収に与える、各種原料の影響を調べた研究を見ることができる。
DAL POZZO A.らは、ヒト死体から採取した皮膚を利用して、パラベン類の皮膚透過性が溶媒や基材となる乳化タイプによってどのような影響を受けるか調べている。
・ Dal Pozzo A (Inst.di Chim.Biochim.G.Ronzani) : Percutaneous absorption of parabens from cosmetic formulation , Int.J.Cosmetic Science , 18 , 57-66 , (1996)

また、国立衛研の徳永らは、モルモットの剥離皮膚を用いて、メチルパラベン、エチルパラベン、サリチル酸等の皮膚透過性に、各種界面活性剤がどのような影響を与えるかを検討し、これらの透過速度とある種の刺激性試験との間には、よい相関が見られたことを報告している。
・徳永裕司(国立衛研)ら:エチルパラベンを透過指標物質とする界面活性剤のモルモットの剥離皮膚への影響, 香粧会誌, Vol.21, No.2, 114-120, (1997)
・徳永裕司(国立衛研)ら:アニオン性およびカチオン性界面活性剤によるメチルパラベンのモルモットの皮膚透過への影響, 香粧会誌, Vol.18, No.2, 127-132, (1994)
・徳永裕司(国立衛研)ら:非イオン性界面活性剤によるメチルパラベンのモルモットの皮膚透過への影響, 香粧会誌, Vol.19, No.2, 112-117, (1995)
・徳永裕司(国立衛研)ら:サリチル酸を透過指標物質とする界面活性剤のモルモットの剥離皮膚への影響, 医薬品研究, Vol.27, 606-612, (1996)

その他、資生堂の田中らは、ヘアレスマウスの皮膚を用い、2-ヒドロキシプロピル-β-シクロデキストリンが、メチルパラベンの皮膚透過性や加水分解の受け易さに影響を与えていることを明らかにした。
・田中宗男((株)資生堂)ら:2-ヒドロキシプロピル-β

-シクロデキストリンによるパラベンの In vitro 皮膚透過挙動, 香粧会誌, Vol.17, No.4, 185-190, (1993)
・ Matsuda H.(Shiseido Research Labs.) : Inclusion Complexation of p-Hydroxybenzoic Acid Esters with 2-Hydroxypropyl-β-cyclodextrins. On Changes in Solubility and Antimicrobial Activity , Chem.Pharm.Bull., 41(8), 1448-1452, (1993)

以上。

COMMISSION DIRECTIVE 97/18/EC

of 17 April 1997

postponing the date after which animal tests are prohibited for ingredients or combinations of ingredients of cosmetic products

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 76/768/EEC of 27 July 1996 on the approximation of the laws of the Member States relating to cosmetic products (1), as last amended by Commission Directive 97/1/EC (2), and in particular Article 4 (1) (i) thereof;

After consulting the Scientific Committee on Cosmetology,

Whereas the main objective of Directive 76/768/EEC is to protect public health; whereas, to this end, it is indispensable to carry out the certain toxicological tests to evaluate the safety for human health of ingredients and combinations of ingredients used in cosmetic product formulations,

Whereas pursuant to Article 4 (1) (i) of Directive 76/768/EEC Member States must ban the placing on the market of cosmetic products containing ingredients or combinations of ingredients tested on animals after 1 January 1998 in order to meet the requirements of the Directive;

Whereas the second sentence of this provision provides that the Commission shall submit draft measures to postpone the date of implementation if there has been insufficient progress in developing satisfactory methods to replace animal testing, and in particular in those cases where alternative methods of testing, despite all reasonable endeavours, have not been scientifically validated as offering an equivalent level of protection for the consumer, taking into account OECD toxicity test guidelines;

Whereas progress has been made in research into alternative methods of testing, in particular in the fields of percutaneous absorption and local risks to the eyes and skin; whereas it has not yet been possible to validate scientifically any alternative testing method; whereas the OECD has not yet adopted pertinent guidelines for toxicity tests in the field of alternative testing methods;

Whereas it is unlikely that the state of the art will change before 1 January 1998; whereas, therefore, the date provided for in Article 4 (1) (i) of Directive 76/768/EEC should be postponed, in compliance with the second sentence of this provision;

Whereas Directive 76/768/EEC provides that the date be postponed for a sufficient period, and in any case for no less than two years; whereas, therefore, it is necessary to stipulate a date later than 1 January 2000; whereas at this stage it is extremely difficult to foresee the date by which certain alternative methods for testing certain ingredients or combinations of ingredients for the presence of certain risks for human health will have been scientifically validated;

Whereas, however, it can be foreseen that alternative methods will progressively become available in regard to percutaneous absorption, photoirritation, eye irritation and skin irritation;

Whereas, likewise, taking into account the provision's objective, scientific reassessment should not be excessively delayed; whereas, therefore, it is necessary to lay down at this stage a date before which it can be foreseen that no alternative method of testing will have been adequately scientifically validated;

Whereas it is therefore appropriate to postpone the date to 30 June 2000;

Whereas, in these circumstances, it is not possible to lay down a time limit offering the certainty that it will be possible to implement the ban on animal experiments on a specified date; whereas, therefore, the Commission is not in a position to exercise its powers under Article 4 (1) (i) of the Directive except in part;

Whereas it is therefore necessary to provide that the Commission shall submit new draft measures under the conditions provided for in this Article;

Whereas postponement of the date shall not be prejudicial to the objective of reducing the number of test animals and their suffering wherever possible, notably through the use of screening tests;

Whereas everything must be done to ensure that alternative methods to animal experiments are developed, validated and accepted; whereas, pursuant to the provisions of Article 130f (3) of the Treaty and the Fourth Framework Programme for Research, the Commission must take the necessary measures to promote research into and the validation of alternative methods to animal experiments in the field of ingredients and combinations of ingredients used in cosmetic product formulations;

(1) OJ No L 262, 27. 9. 1976, p. 169.
(2) OJ No L 16, 18. 1. 1997, p. 85.

Whereas the measures provided for in this Directive are in accordance with the opinion of the Committee on the Adaptation to Technical Progress of the Directives on the Removal of Technical Barriers to Trade in the Cosmetic Products Sector,

HAS ADOPTED THIS DIRECTIVE:

Article 1

The date of '1 January 1998' shall be replaced by '30 June 2000' in the first sentence of Article 4 (1) (i) of Directive 76/768/EEC.

Article 2

If there has been insufficient progress in developing satisfactory methods to replace animal testing, and in particular in those cases where alternative methods of testing, despite all reasonable endeavours, have not been scientifically validated as offering an equivalent level of protection for the consumer, taking into account OECD toxicity test guidelines, the Commission shall, by 1 January 2000, submit draft measures to postpone the date referred to in Article 1 for those testing methods in respect of which there has been insufficient progress in developing alternative methods, in accordance with the procedure laid down in Article 10 of Directive 76/768/EEC. Before submitting such measures, the Commission will consult the Scientific Committee on Cosmetology.

Article 3

1. Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with this Directive no later than 31 December 1997. They shall forthwith inform the Commission thereof.

When Member States adopt these provisions, these shall contain a reference to this Directive or shall be accompanied by such reference at the time of their official publication. The procedure for such reference shall be adopted by Member States.

2. Member States shall communicate to the Commission the provisions of national law which they adopt in the field covered by this Directive.

Article 4

This Directive shall enter into force on the third day following its publication in the *Official Journal of the European Communities.*

Article 5

This Directive is addressed to the Member States.

Done at Brussels, 17 April 1997.

For the Commission

Emma BONINO

Member of the Commission

添付資料-2

European Commission
Joint Research Centre
Environment Institute
21020 Ispra (VA)
Italy

STATEMENT ON THE SCIENTIFIC VALIDITY OF THE EPISKIN™ TEST (AN *IN VITRO* TEST FOR SKIN CORROSIVITY)

At its 10th meeting, held on 31 March 1998 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

> The results obtained with the EPISKIN™ test (involving the use of a reconstructed human skin model) in the ECVAM international validation study on *in vitro* tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The EPISKIN test proved applicable to testing a diverse group of chemicals of different physical forms, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/ surfactants. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good. The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied; it was also able to distinguish between known R35 (UN[2] packing group I) and R34 (UN packing groups II & III) chemicals. The Committee therefore agrees with the conclusion from this formal validation study that the EPISKIN test is scientifically validated for use as a replacement for the animal test, and that it is ready to be considered for regulatory acceptance.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results and evaluation of the validation study by the Management Team, which is to be published in *Toxicology in Vitro*.[3]

This validation study was conducted in accordance with the general principles laid down in the report of the CAAT[2]/ERGATT[2] workshop held in 1990,[4] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,[5] criteria laid down by ECVAM and the ECB,[2,6] criteria recommended at an OECD[2] workshop held in 1996,[7] and the US ICCVAM[2] report on validation and regulatory acceptance.[8] The outcome of a prevalidation study on *in vitro* tests for skin corrosivity was published in 1995, as ECVAM workshop report 6.[9] A separate report on the selection of the test chemicals for the validation study is to be published alongside the Management Team's report in *Toxicology in Vitro*.[10]

Michael Balls
Head of Unit
ECVAM
JRC Environment Institute
European Commission
Ispra

Guy Corcelle
Head of Unit
DGXI/E/2
European Commission
Brussels

3 April 1998

1. The ESAC was established by the European Commission, and is composed of representatives of the EU Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 31 March 1998:

Dr B Blaauboer (ERGATT)
Professor J Castell (Spain)
Dr B Garthoff (EFPIA)
Dr C Hendriksen (The Netherlands)
Professor G Papadopoulos (Greece)
Dr B Rusche (Eurogroup for Animal Welfare)
Professor H Spielmann (Germany)
Professor H. Tritthart (Austria)
Professor E Walum (Sweden)

Dr P Botham (ECETOC)
Dr D Clark (UK)
Professor A Guillouzo (France)
Dr R Lorenzini (Italy)
Professor V Rogiers (Belgium)
Dr O de Silva (COLIPA)
Dr O Svendsen (Denmark)
Dr M Viluksela (Finland)

Professor M Balls (ECVAM)
Dr J Fentem (ECVAM)
Ms S Louhimies (DGXI)
Mr A Van Elst (DGXXIV)

Mr G Corcelle (DGXI)
Dr G Fracchia (DGXII)
Dr M Robert (DGIII)

2. CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; ECB: European Chemicals Bureau, Ispra, Italy; ERGATT: European Research Group for Alternatives in Toxicity Testing, Utrecht, The Netherlands; ICCVAM: *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; OECD: Organization for Economic Cooperation and Development, Paris, France; UN: United Nations.

3. Fentem JH, Archer GEB, Balls M, Botham PA, Curren RD, Earl LK, Esdaile DJ, Holzhütter H-G & Liebsch M (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. *Toxicology in Vitro*, in press.

4. Balls M, Blaauboer B, Brusick D, Frazier J, Lamb D, Pemberton M, Reinhardt C, Roberfroid M, Rosenkranz H, Schmid B, Spielmann H, Stammati AL & Walum E (1990) Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA* 18: 303-337.

5. Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H & Zucco F (1995) Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. *ATLA* 23: 129-147.

6. Balls M & Karcher W (1995) The validation of alternative test methods. *ATLA* 23: 884-886.

7. Anon. (1996) *Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods.* 60pp. Paris: OECD.

8. Anon. (1997) *Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods.* 105pp. Research Triangle Park, NC: NIEHS.

9. Botham PA, Chamberlain M, Barratt MD, Curren RD, Esdaile DJ, Gardiner JR, Gordon VC, Hildebrand B, Lewis RW, Liebsch M, Logemann P, Osborne R, Ponec M, Régnier J-F, Steiling W, Walker AP & Balls M (1995) A prevalidation study on *in vitro* skin corrosivity testing. The report and recommendations of ECVAM workshop 6. *ATLA* 23: 219-255.

10. Barratt MD, Brantom PG, Fentem JH, Gerner I, Walker AP & Worth AP (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. *Toxicology in Vitro*, in press.

General information about the ECVAM skin corrosivity validation study:

A. The study was coordinated from ECVAM, and the Management Team (MT) was chaired by Dr Julia Fentem (ECVAM). The other four MT members acted as representatives of the "lead laboratories" and each took responsibility for one of the four tests included in the validation study: Dr Rodger Curren (Microbiological Associates Inc., USA; CORROSITEX™), Dr Lesley Earl (Unilever, UK; rat skin TER assay), Mr David Esdaile (Rhône-Poulenc Agro, France; EPISKIN™), and Dr Manfred Liebsch (ZEBET, Germany; Skin²™ assay). The study was principally funded by ECVAM, under the terms of 14 separate contracts with the participating organisations. Professor Michael Balls (ECVAM) and Dr Philip Botham (ESAC; ZENECA CTL, UK) represented the sponsors in any contacts with the MT. In addition to ECVAM, the participating organisations were: Agence du Medicament (France), BASF Aktiengesellschaft (Germany), BIBRA International (UK), COVANCE (UK), Humboldt University (Germany), Huntingdon Life Sciences (UK), INRS (France), Microbiological Associates Inc. (USA), Microbiological Associates Ltd (UK), Rhône-Poulenc Agro (France), Sanofi Recherche (France), Unilever Research (UK), ZEBET, BgVV (Germany) and ZENECA CTL (UK).

B. This study began in 1996, as a follow-up to a prevalidation study on *in vitro* tests for replacing the *in vivo* Draize rabbit test for skin corrosivity. The main objectives were to: (a) identify tests capable of discriminating corrosives (C) from non-corrosives (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only); and (b) determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. The tests selected for inclusion in the validation study were: (a) the rat skin TER assay; (b) CORROSITEX™; (c) the Skin²™ ZK1350 corrosivity test; and (d) EPISKIN™. Each test was conducted in three independent laboratories, according to the principles, criteria and procedures for undertaking validation studies outlined previously by ECVAM in conjunction with international experts in this area. Prediction models for the four tests were clearly defined in the test protocols.

C. A test set of 60 chemicals was selected by an independent Chemicals Selection Sub-Committee, including organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC) and soaps/surfactants (3NC). The first set of ten coded chemicals was distributed independently of the MT and participating laboratories in June 1996. Further to the satisfactory completion of the first phase of the study, the remaining 50 coded chemicals were distributed in September 1996. The results obtained were submitted to ECVAM's statistician, Dr Graeme Archer, for independent analysis in consultation with Dr Hermann-Georg Holzhütter (Humboldt University, Berlin, Germany). Data analysis and preparation of the final reports took place between May and October 1997.

D. EPISKIN™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin corrosivity testing involves topical application of test materials to the surface of the skin for 3, 60 and 240 min, and the subsequent assessment of their effects on cell viability by using the MTT assay. An in-house evaluation and prevalidation of the test was conducted during 1994-96. On the basis of these studies, the test protocol was refined prior to its inclusion in this validation study.

EPISKIN Prediction Model:

Treatment time (min)	Viability (%)	C/NC	EU risk phrase	UN packing group
3	< 35	C	R35	I
3 / 60	≥35 / <35	C	R34	II
60 / 240	≥35 / <35	C	R34	III
240	≥ 35	NC	no label	-

E. The prediction model for the EPISKIN test was used to classify the corrosivity potentials of the 60 test chemicals on the basis of the *in vitro* data obtained in the three laboratories conducting the test. Comparing these *in vitro* classifications with the *in vivo* classifications independently assigned to the chemicals before the blind trial began gave the following key statistical parameters:

Sensitivity:	C	83%
	R34/II & III	75%
	R35/I	39%
Specificity:		80%
Predictivity:	C	77%
	R34/II & III	64%
	R35/I	53%
Accuracy:	C/NC	81%
	R35/R34/NC	74%

The underprediction and overprediction rates for the EPISKIN test relative to the study objectives were:

Objective (a): C *v* NC	underprediction rate	17%
	overprediction rate	20%

Objective (b): R35/I *v* R34/II & III *v* NC

underprediction rate	
R35/I → NC	17%
R34/II & III → NC	18%
overprediction rate	
NC → R35/I	1%
NC → R34/II & III	19%
R34/II & III → R35/I	8%

F. In order for the EPISKIN test to be considered for use for legislative and other purposes, measures will be taken to press for the updating of OECD Testing Guideline 404 and Annex V method B.4 of *Directive 67/548/EEC.*

G. A statement on the scientific validity of the rat skin transcutaneous electrical resistance (TER) assay for skin corrosivity testing was also endorsed by the ESAC on 31 March 1998. The two other methods included in the validation study, CORROSITEX and Skin^2, did not meet all of the criteria for them to be considered acceptable as replacement tests. The corrosivity potentials of about 40% of the test chemicals could not be assessed with CORROSITEX, although it may be valid for testing specific classes of chemicals (such as organic bases and inorganic acids). The Skin^2 assay, as conducted in this validation study, had an unacceptably high underprediction rate (57%), although it had a specificity of 100%. It is recognised that both of these methods could be useful if they were incorporated into a tiered testing strategy for skin corrosivity.

添付資料-3

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STATEMENT ON THE SCIENTIFIC VALIDITY OF THE RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST (AN *IN VITRO* TEST FOR SKIN CORROSIVITY)

At its 10th meeting, held on 31 March 1998 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

> The results obtained with the rat skin transcutaneous electrical resistance (TER) test in the ECVAM international validation study on *in vitro* tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The rat skin TER test proved applicable to testing a diverse group of chemicals of different physical forms, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/surfactants. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good. The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied. The Committee therefore agrees with the conclusion from this formal validation study that the rat skin TER test is scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and non-corrosive chemicals, and that this test is ready to be considered for regulatory acceptance.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results and evaluation of the validation study by the Management Team, which is to be published in *Toxicology in Vitro*.[3]

This validation study was conducted in accordance with the general principles laid down in the report of the CAAT[2]/ERGATT[2] workshop held in 1990,[4] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,[5] criteria laid down by ECVAM and the ECB,[2,6] criteria recommended at an OECD[2] workshop held in 1996,[7] and the US ICCVAM[2] report on validation and regulatory acceptance.[8] The outcome of a prevalidation study on *in vitro* tests for skin corrosivity was published in 1995, as ECVAM workshop report 6.[9] A separate report on the selection of the test chemicals for the validation study is to be published alongside the Management Team's report in *Toxicology in Vitro*.[10]

Michael Balls
Head of Unit
ECVAM
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Guy Corcelle
Head of Unit
DGXI/E/2
European Commission
Brussels

3 April 1998

1. The ESAC was established by the European Commission, and is composed of representatives of the EU Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 31 March 1998:

Dr B Blaauboer (ERGATT)
Professor J Castell (Spain)
Dr B Garthoff (EFPIA)
Dr C Hendriksen (The Netherlands)
Professor G Papadopoulos (Greece)
Dr B Rusche (Eurogroup for Animal Welfare)
Professor H Spielmann (Germany)
Professor H. Tritthart (Austria)
Professor E Walum (Sweden)

Dr P Botham (ECETOC)
Dr D Clark (UK)
Professor A Guillouzo (France)
Dr R Lorenzini (Italy)
Professor V Rogiers (Belgium)
Dr O de Silva (COLIPA)
Dr O Svendsen (Denmark)
Dr M Viluksela (Finland)

Professor M Balls (ECVAM)
Dr J Fentem (ECVAM)
Ms S Louhimies (DGXI)
Mr A Van Elst (DGXXIV)

Mr G Corcelle (DGXI)
Dr G Fracchia (DGXII)
Dr M Robert (DGIII)

2. CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; ECB: European Chemicals Bureau, Ispra, Italy; ERGATT: European Research Group for Alternatives in Toxicity Testing, Utrecht, The Netherlands; ICCVAM: *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; OECD: Organization for Economic Cooperation and Development, Paris, France; UN: United Nations.

3. Fentem JH, Archer GEB, Balls M, Botham PA, Curren RD, Earl LK, Esdaile DJ, Holzhütter H-G & Liebsch M (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. *Toxicology in Vitro*, in press.

4. Balls M, Blaauboer B, Brusick D, Frazier J, Lamb D, Pemberton M, Reinhardt C, Roberfroid M, Rosenkranz H, Schmid B, Spielmann H, Stammati AL & Walum E (1990) Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA* 18: 303-337.

5. Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H & Zucco F (1995) Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. *ATLA* 23: 129-147.

6. Balls M & Karcher W (1995) The validation of alternative test methods. *ATLA* 23: 884-886.

7. Anon. (1996) *Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods*. 60pp. Paris: OECD.

8. Anon. (1997) *Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods.* 105pp. Research Triangle Park, NC: NIEHS.

9. Botham PA, Chamberlain M, Barratt MD, Curren RD, Esdaile DJ, Gardiner JR, Gordon VC, Hildebrand B, Lewis RW, Liebsch M, Logemann P, Osborne R, Ponec M, Régnier J-F, Steiling W, Walker AP & Balls M (1995) A prevalidation study on *in vitro* skin corrosivity testing. The report and recommendations of ECVAM workshop 6. *ATLA* 23: 219-255.

10. Barratt MD, Brantom PG, Fentem JH, Gerner I, Walker AP & Worth AP (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. *Toxicology in Vitro*, in press.

General information about the ECVAM skin corrosivity validation study:

A. The study was coordinated from ECVAM, and the Management Team (MT) was chaired by Dr Julia Fentem (ECVAM). The other four MT members acted as representatives of the "lead laboratories" and each took responsibility for one of the four tests included in the validation study: Dr Rodger Curren (Microbiological Associates Inc., USA; CORROSITEX™), Dr Lesley Earl (Unilever, UK; rat skin TER assay), Mr David Esdaile (Rhône-Poulenc Agro, France; EPISKIN™), and Dr Manfred Liebsch (ZEBET, Germany; Skin^{2}™ assay). The study was principally funded by ECVAM, under the terms of 14 separate contracts with the participating organisations. Professor Michael Balls (ECVAM) and Dr Philip Botham (ESAC; ZENECA CTL, UK) represented the sponsors in any contacts with the MT. In addition to ECVAM, the participating organisations were: Agence du Medicament (France), BASF Aktiengesellschaft (Germany), BIBRA International (UK), COVANCE (UK), Humboldt University (Germany), Huntingdon Life Sciences (UK), INRS (France), Microbiological Associates Inc. (USA), Microbiological Associates Ltd (UK), Rhône-Poulenc Agro (France), Sanofi Recherche (France), Unilever Research (UK), ZEBET, BgVV (Germany) and ZENECA CTL (UK).

B. This study began in 1996, as a follow-up to a prevalidation study on *in vitro* tests for replacing the *in vivo* Draize rabbit test for skin corrosivity. The main objectives were to: (a) identify tests capable of discriminating corrosives (C) from non-corrosives (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only); and (b) determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. The tests selected for inclusion in the validation study were: (a) the rat skin TER assay; (b) CORROSITEX™; (c) the Skin^{2}™ ZK1350 corrosivity test; and (d) EPISKIN™. Each test was conducted in three independent laboratories, according to the principles, criteria and procedures for undertaking validation studies outlined previously by ECVAM in conjunction with international experts in this area. Prediction models for the four tests were clearly defined in the test protocols.

C. A test set of 60 chemicals was selected by an independent Chemicals Selection Sub-Committee, including organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC) and soaps/surfactants (3NC). The first set of ten coded chemicals was distributed independently of the MT and participating laboratories in June 1996. Further to the satisfactory completion of the first phase of the study, the remaining 50 coded chemicals were distributed in September 1996. The results obtained were submitted to ECVAM's statistician, Dr Graeme Archer, for independent analysis in consultation with Dr Hermann-Georg Holzhütter (Humboldt University, Berlin, Germany). Data analysis and preparation of the final reports took place between May and October 1997.

D. The rat skin TER assay has been used successfully as a routine in-house test for several years. When used in screening mode, the TER method is employed to predict corrosivity potential rather than the degree of corrosive effect (i.e. potency), and it has been used primarily to guide humane *in vivo* skin testing. The TER assay has been evaluated in several intralaboratory and interlaboratory studies, and it performed creditably in the prevalidation study conducted during 1993 and 1994. The test protocol evaluated in this validation study had been refined on the basis of recommendations from the prevalidation study, to include a dye binding procedure for reducing the number of false positive predictions obtained previously with test materials containing surfactants and solvents. In outline, test materials are applied for up to 24 hr to the epidermal surfaces of skin discs taken from the pelts of humanely killed young rats. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a predetermined threshold level (5kΩ).

Rat Skin TER Assay Prediction Model:

TER (kΩ)	Treatment time (hours)	Mean disc dye content	C/NC	EU risk phrase	UN packing group
> 5	2 & 24	NM[a]	NC	no label	-
≤ 5	2	-	C	R35	I
	24	-	C	R34	II/III
Surfactants/neutral organics:					
≤ 5	24	≥ +ve control	C	R34	II/III
	24	< +ve control	NC	no label	-

[a]NM = not measured

E. The prediction model for the rat skin TER test was used to classify the corrosivity potentials of the 60 test chemicals on the basis of the *in vitro* data obtained in the three laboratories conducting the test. Comparing these *in vitro* classifications with the *in vivo* classifications independently assigned to the chemicals before the blind trial began gave the following key statistical parameters:

Sensitivity:	C	88%
	R34/II & III	18%
	R35/I	88%
Specificity:		72%
Predictivity:	C	72%
	R34/II & III	40%
	R35/I	22%
Accuracy:	C/NC	79%
	R35/R34/NC	55%

The underprediction and overprediction rates for the TER test relative to the study objectives were:

Objective (a): C *v* NC	underprediction rate	12%
	overprediction rate	28%

Objective (b): R35/I *v* R34/II & III *v* NC	underprediction rate	
	R35/I → NC	6%
	R34/II & III → NC	14%
	overprediction rate	
	NC → R35/I	12%
	NC → R34/II & III	16%
	R34/II & III → R35/I	69%*

*unacceptable according to the criteria defined by the MT before undertaking the data analysis

F. In order for the rat skin TER test to be considered for use for legislative and other purposes, measures will be taken to press for the updating of OECD Testing Guideline 404 and Annex V method B.4 of *Directive 67/548/EEC*.

G. A statement on the scientific validity of the EPISKIN™ assay for skin corrosivity testing was also endorsed by the ESAC on 31 March 1998. The two other methods included in the validation study, CORROSITEX and Skin^2, did not meet all of the criteria for them to be considered acceptable as replacement tests. The corrosivity potentials of about 40% of the test chemicals could not be assessed with CORROSITEX, although it may be valid for testing specific classes of chemicals (such as organic bases and inorganic acids). The Skin^2 assay, as conducted in this validation study, had an unacceptably high underprediction rate (57%), although it had a specificity of 100%. It is recognised that both of these methods could be useful if they were incorporated into a tiered testing strategy for skin corrosivity.

添付資料-4

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STATEMENT ON THE APPLICATION OF THE 3T3 NRU PT TEST TO UV FILTER CHEMICALS

At its 10th meeting, held on 31 March 1998 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

> The outcome of the study with UV filter chemicals further confirms the validity of the 3T3 NRU PT test, which has now been demonstrated to be applicable for testing these types of chemicals for their phototoxic potential.

The ESAC has been regularly kept informed of the progress of the special study, and this endorsement was based on the assessment of various documents and a verbal report to the ESAC by Professor Horst Spielmann, Chairman of the Management Team for the study.

This special study was conducted in accordance with the general principles for validation laid down in the report of the CAAT[2]/ERGATT[2] workshop held in 1990,[3] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,[4] criteria laid down by ECVAM and the ECB,[2,5] criteria recommended at an OECD[2] workshop held in 1996,[6] and the US ICCVAM[2] report on validation and regulatory acceptance.[7]

A detailed report on the study will be published in *ATLA* during 1998,[8] and the report on Phase II of the EU/COLIPA validation study on the 3T3 NRU PT test will shortly be published in *Toxicology in Vitro*.[9] The ESAC formally endorsed the method as a scientifically validated test at its meeting on 1-2 October 1997.[10]

The experience and results obtained during the study on UV filters have been taken into account in the drafting of a proposed test guideline on the *in vitro* 3T3 NRU PT test.

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Guy Corcelle
Head of Unit
DGXI/E/2
European Commission
Brussels

20 May 1998

1. The ESAC was established by the European Commission, and is composed of representatives of the EU Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 31 March 1998:

Dr B. Blaauboer (ERGATT)
Professor J. Castell (Spain)
Dr B. Garthoff (EFPIA)
Dr C. Hendriksen (The Netherlands)
Professor G. Papadopoulos (Greece)
Dr B. Rusche (Eurogroup for Animal Welfare)
Professor H. Spielmann (Germany)
Professor H. Tritthart (Austria)
Professor E. Walum (Sweden)

Dr P. Botham (ECETOC)
Dr D. Clark (UK)
Professor A. Guillouzo (France)
Dr R. Lorenzini (Italy)
Professor V. Rogiers (Belgium)
Dr O. de Silva (COLIPA)
Dr O. Svendsen (Denmark)
Dr M. Viluksela (Finland)

Professor M. Balls (ECVAM)
Dr J. Fentem (ECVAM)
Ms S. Louhimies (DGXI)
Mr A. Van Elst (DGXXIV)

Mr G. Corcelle (DGXI)
Dr G. Fracchia (DGXII)
Dr M. Robert (DGIII)

2. CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; ECB: European Chemicals Bureau, Ispra, Italy; ERGATT: European Research Group for Alternatives in Toxicity Testing, Utrecht, The Netherlands; ICCVAM: *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; OECD: Organization for Economic Cooperation and Development, Paris, France.

3. Balls M, Blaauboer B, Brusick D, Frazier J, Lamb D, Pemberton M, Reinhardt C, Robertfroid M, Rosenkranz H, Schimid B, Spielmann H, Stammati AL & Walum E (1990). Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA* 18: 303-337.

4. Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H & Zucco F (1995). Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. *ATLA* 23: 129-147.

5. Balls M & Karcher W (1995). The validation of alternative test methods. *ATLA* 23: 884-886.

6. Anon. (1996). Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. 60pp. Paris: OECD.

7. Anon. (1997). Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods. 105pp. Research Triangle Park, NC: NIEHS.

8. Spielmann H, Balls M, Dupuis J, Pape WJW, de Silva O, Holzhütter HG, Gerberick F, Liebsch M, Lovell WW & Pfannenbecker U (1998). A study on UV filter chemicals from Annex VII of the EU Cosmetics Directive 76/768 in the *in vitro* 3T3 NRU phototoxicity test. *ATLA* 26, in press.

9. Spielmann H, Balls M, Dupuis J, Pape WJW, Pechovitch G, de Silva O, Holzhütter HG, Clothier R, Desolle P, Gerberick F, Liebsch M, Lovell WW, Maurer T, Pfannenebecker U, Polthast JM, Csato M, Sladowski D, Steiling W & Brantom P (1998). The international EU/COLIPA *in vitro* phototoxicity validation study: results of phase II (blind trial). Part 1: the 3T3 NRU phototoxicity test. *Toxicology in Vitro* 12, 305-327.

10. Anon (1998). Statement on the scientific validation of the 3T3 NRU PT test (an *in vitro* test for phototoxic potential). *ATLA* 26, 7-8.

3

OECD GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

In Vitro 3T3 NRU Phototoxicity Test

INTRODUCTION

1. Phototoxicity (photoirritation) is defined as a toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical.

2. Information derived from the *in vitro* 3T3 NRU phototoxicity test serves to identify the phototoxic potential of a test substance, i.e. the existence or absence of possible hazards likely to arise from a test substance in association with exposure to UV and visible light.

3. Since the toxicological endpoint of the *in vitro* test is determination of *photocytotoxicity*, induced by the combined action of a chemical and light, compounds that are phototoxic *in vivo* after systemic application and distribution to the skin, as well as compounds that act as photoirritants after topical application to the skin, can be identified by the test.

4. The *in vitro* 3T3 NRU phototoxicity test was developed and validated in a joint EU/COLIPA project from 1992-1997 (1-3), to establish a valid *in vitro* alternative to the various *in vivo* tests in use, none of which has been accepted by the OECD. In 1996 an OECD workshop recommended an *in vitro* tier testing approach for phototoxicity assessment (4).

5. Results from the *in vitro* 3T3 NRU phototoxicity test were compared with *acute* phototoxicity / photoirritation effects *in vivo* in animals and humans, and the test has been shown to give excellent predictivity for these effects. The test is not designed to predict other adverse effects that may arise from the combined action of a chemical and light, e.g. *photogenotoxicity*, *photoallergy*, and *photocarcinogenicity*, although many chemicals which show these specific properties will react positive in the *in vitro* 3T3 NRU phototoxicity test. In addition, the test is not designed to permit an assessment of *phototoxic potency*.

6. Definitions used in this Guideline are set out in Annex 1.

7. A sequential approach to phototoxicity testing of chemicals is set out in Annex 2.

INITIAL CONSIDERATION

8. Many types of chemicals have been reported to induce phototoxic effects (5-8). The only common feature is their ability to absorb light energy within the sunlight region. According to the first law of photochemistry (Grotthaus-Draper's Law) photoreaction requires sufficient absorption of light quanta. Thus, before biological testing according to the present test guideline is considered, a UV/vis absorption spectrum of the test chemical should be determined according to OECD Test Guideline 101. If the molar extinction / absorption coefficient is less than 10 litre × mol^{-1} × cm^{-1}, the chemical has no photoreactive potential and does not need to be tested in the *in vitro* 3T3 NRU phototoxicity test or any other biological test for adverse photochemical effects (see Annex 2).

PRINCIPLE OF THE TEST METHOD

9. Four mechanisms have been identified by which absorption of light by a (chemical) chromophore can result in a phototoxic response. All of them result in cell damage. Therefore, the *in vitro* 3T3 NRU phototoxicity test is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of UVA/vis light. Cytotoxicity in this test is expressed as a concentration dependent reduction of the uptake of the vital dye, Neutral Red (NR; 9) 24 hours after treatment with the test chemical and irradiation.

10. Balb/c 3T3 cells are maintained in culture for 24 h for the formation of monolayers. Two 96-well plates per test chemical are then preincubated with eight different concentrations of the chemical for 1 h. Thereafter one of the two plates is exposed to a non-cytotoxic UVA/vis light dose of 5 J/cm² UVA (+UV experiment), whereas the other plate is kept in the dark (-UV experiment). In both plates, the treatment medium is then replaced by culture medium and after another 24 h of incubation, cell viability is determined by Neutral Red Uptake (NRU) for 3 h. Cell viability, expressed as percentage of untreated negative controls, is calculated for each of the eight test concentrations. To predict the phototoxic potential, the concentration responses obtained in the presence (+UV) and in the absence (-UV) of irradiation are compared, usually at the EC_{50} level, i.e. at the concentration inhibiting cell viability by 50 % cf. untreated controls.

DESCRIPTION OF THE TEST METHOD

Preparations

Cells

11. A permanent mouse fibroblast cell line - Balb/c 3T3, clone 31 - either from ATCC or from ECACC was used in the validation study, and is therefore recommended. Other cells or cell lines may be successfully used with the same test protocol, if the culture conditions are adapted to the specific needs of the cells, but equivalency must be demonstrated.

12. Cells should be checked regularly for the absence of mycoplasma contamination and should only be used if the results of such checking was satisfactory.

13. Since the UVA sensitivity of cells may increase with the number of passages, Balb/c 3T3 cells of the lowest obtainable passage number should be used, preferably less than 100. It is important that UVA sensitivity of the Balb/c 3T3 cells is regularly checked according to the quality control procedure described in this Guideline.

Media and culture conditions

14. Appropriate culture media and incubation conditions should be used for routine cell passage and during the test procedure. For Balb/c 3T3 cells, these are DMEM supplemented with 10% new-born calf serum, 4 mM Glutamine, Penicillin and Streptomycin, and humidified incubation at 37°C / 7.5% CO_2. It is particularly important that cell culture conditions assure a cell cycle time within the normal historical range of the cells or cell line used.

Preparation of cultures

15. Cells from frozen stock cultures are seeded in culture medium at an appropriate density and subcultured at least once before they are used in the *in vitro* 3T3 NRU phototoxicity test.

16. For the phototoxicity test cells are seeded in culture medium at a density such that cultures will not reach confluence by the end of the test, i.e. when cell viability is determined 48 h after the seeding of the cells. For Balb/c 3T3 cells grown in 96-well plates, 1×10^4 cells per well is the recommended cell density.

17. For each test chemical, cells are seeded identically in two separate 96-well plates, which are then taken concurrently through the whole test procedure under identical culture conditions, except for the time period where one of the plates is irradiated (+UVA/vis) and the other one is kept in the dark (-UVA/vis).

Preparation of test chemicals

18. Test chemicals must be freshly prepared immediately prior to use, unless stability data demonstrate the acceptability of storage. Preparation under red light may be required when rapid photodegradation is likely to occur.

19. Test chemicals should be dissolved in buffered salt solutions, e.g. Earl's Balanced Salt Solution, (EBSS) or Phosphate Buffered Saline (PBS), which, to avoid interference during irradiation, must be free from protein components and light absorbing pH indicator colours.

20. Test chemicals of limited solubility in water should be dissolved in appropriate solvents at 100-fold the desired final concentration and then diluted 1:100 with the buffered salt solution. If a solvent is used it must be present at a constant volume of 1% (v/v) in all cultures, i.e. in the negative controls as well as in all concentrations of the test chemical.

21. Dimethylsulphoxide (DMSO) and ethanol (ETOH) are the recommended solvents. Other solvents of low cytotoxicity (e.g. acetone) may be appropriate, but they should carefully be assessed for specific properties, e.g. reaction with the test chemical, quenching of the phototoxic effect, radical catching properties.

22. Vortex mixing and / or sonication and / or warming to 37°C may be used, if necessary, to aid solubilization.

Preparation of UV irradiation

23. *Light source*: the choice of an appropriate light source and appropriate filtering is the most crucial factor in phototoxicity testing. UVA and visible regions are usually associated with photosensitization (7, 10), whereas UVB is of less relevance and is directly highly cytotoxic, increasing its cytotoxicity through 1000 fold from 313 to 280 nm (11). Criteria for the choice of an appropriate light source should include the essential requirement that the light source emits wavelengths absorbed by the test chemical and that the dose of light (achievable in a reasonable time) should be sufficient for the detection of known photosensitizers. Furthermore, the wavelengths and doses employed should not be unduly deleterious to the test system, which includes the emission of heat (infra red region).

24. The simulation of sunlight with solar simulators is considered the optimal light source. Both, Xenon arcs and (doped) mercury-metal halide arcs are used in solar simulators. The latter have the advantage of emitting less heat and of being cheaper, but the match to sunlight is not perfect. Since all solar simulators emit significant quantities of UVB, they should be suitably filtered to attenuate the highly cytotoxic UVB wavelengths.

25. For the *in vitro* 3T3 NRU phototoxicity test an irradiance spectrum practically devoid of UVB should be used (UVA:UVB ~ 1:20). An example of the spectral irradiance distribution of