

新規試験法提案書

*In vitro* 皮膚透過試験

平成26年 1 月

国立医薬品食品衛生研究所

# 新規試験法提案書

平成 26 年 1 月 20 日

No. 2013-03

## *In vitro* 皮膚透過試験に関する提案

平成 25 年 10 月 21 日に東京、国立医薬品食品衛生研究所にて開催された新規試験法評価会議(通称：JaCVAM 評価会議)において以下の提案がなされた。

**提案内容：***In vitro* 皮膚透過試験は、化学物質の皮膚からの透過性を測定するものであり、毒性を評価あるいは予測するものではないが、目的とする物質又は製品の全身曝露量を推定する代替試験法として、行政上利用することは可能である。

この提案書は、OECD (Organisation for Economic Co-operation and Development) Test Guideline 428およびEUのSCCP (Scientific Committee on Consumer Products) opinion on basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients等をもとに、皮膚透過性試験評価委員会によりまとめられた文書を用いてJaCVAM評価会議が評価および検討した結果、その有用性が確認されたことから作成された。

以上の理由により、行政当局の安全性評価方法として「*In vitro*皮膚透過試験」の使用を提案するものである。

吉田武美 

JaCVAM 評価会議 議長

西川秋佳 

西川秋佳

JaCVAM 運営委員会 委員長

## JaCVAM 評価会議

- 吉田武美 (日本毒性学会) : 座長  
浅野哲秀 (日本環境変異原学会)  
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黒澤 努 (日本動物実験代替法学会)  
杉山真理子 (日本化粧品工業連合会)  
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渡部一人 (日本製薬工業協会)

任期：平成 24 年 4 月 1 日～平成 26 年 3 月 31 日

\*: 平成 25 年 4 月 1 日～平成 26 年 3 月 31 日

## JaCVAM 運営委員会

- 西川秋佳 (国立医薬品食品衛生研究所 安全性生物試験研究センター) : 委員長  
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新規試験表評価室) : 事務局

\* : 平成 25 年 8 月 1 日より



**JaCVAM statement  
on skin absorption *in vitro* method**

At the meeting concerning the above method, held on 21 October 2013 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the members of the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board unanimously endorsed the following statement:

**The skin absorption *in vitro* method is considered to be useful to guess the exposed dose of substances or products to whole body for regulatory use.**

Following the review of the results of OECD (Organisation for Economic Co-operation and Development) Test Guideline (TG) 428 and EU の SCCP (Scientific Committee on Consumer Products) opinion on basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients, etc. it is concluded that skin absorption *in vitro* method are clearly beneficial.

The JaCVAM Regulatory Acceptance Board has been regularly kept informed of the progress of the study, and this endorsement is based on an assessment of various documents, including, in particular, the evaluation report prepared by the JaCVAM ad hoc peer review panel for skin absorption testing.



Takemi Yoshida  
Chairperson  
JaCVAM Regulatory Acceptance Board



Akiyoshi Nishikawa  
Chairperson  
JaCVAM Steering Committee

20 January, 2014

The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

This statement was endorsed by the following members of the JaCVAM Regulatory Acceptance Board:

Mr. Takemi Yoshida (Japanese Society of Toxicology): Chairperson  
Mr. Norihide Asano (Japanese Environmental Mutagen Society)  
Mr. Tsutomu Ichiki (Japan Chemical Industry Association)\*  
Mr. Yoshiaki Ikarashi (National Institute of Health Sciences: NIHS)  
Mr. Tsutomu Miki Kurosawa (Japanese Society for Animal Experimentation)  
Mr. Eiji Maki (Japanese Society of Immunotoxicology)  
Mr. Mitsuteru Masuda (nominee by Chairperson)  
Mr. Akiyoshi Nishikawa (NIHS)  
Mr. Yasuo Ohno (nominee by Chairperson)\*  
Mr. Hiroshi Onodera (Pharmaceuticals and Medical Devices Agency)  
Ms. Mariko Sugiyama (Japan Cosmetic Industry Association)  
Ms. Tomoko Tanita (Pharmaceuticals and Medical Devices Agency)\*  
Mr. Takashi Yamada (National Institute of Technology and Evaluation)\*  
Mr. Hiroo Yokozeki (Japanese Society for Dermatoallergology and Contact Dermatitis)  
Ms. Midori Yoshida (NIHS)  
Mr. Isao Yoshimura (nominee by Chairperson)  
Mr. Kazuto Watanabe (Japan Pharmaceutical Manufacturers Association)

Term: From 1st April 2012 to 31st March 2014

\*: From 1st April 2013 to 31st March 2014

This statement was endorsed by the following members of the JaCVAM steering Committee after receiving the report from JaCVAM Regulatory Acceptance Board:

Mr. Akiyoshi Nishikawa (BSRC, NIHS): Chairperson  
Mr. Akihiko Hirose (Division of Risk Assessment, BSRC, NIHS)  
Mr. Masamitsu Honma (Division of Genetics and Mutagenesis, BSRC, NIHS)  
Mr. Jun Kanno (Division of Cellular and Molecular Toxicology, BSRC, NIHS)  
Mr. Toru Kawanishi (NIHS)  
Mr. Kenji Kuramochi (Ministry of Health, Labour and Welfare)\*  
Mr. Toshinari Mitsuoka (Ministry of Health, Labour and Welfare)  
Ms. Kumiko Ogawa (Division of Pathology, BSRC, NIHS)  
Mr. Kazuyuki Saito (Pharmaceutical & Medical Devices Agency)  
Mr. Masahiro Sasaki (Ministry of Health, Labour and Welfare)  
Ms. Yuko Sekino (Division of Pharmacology, BSRC, NIHS)  
Mr. Atsuya Takagi (Animal Management Section of the Division of Cellular and Molecular Toxicology, BSRC, NIHS)  
Mr. Junji Yamamoto (Ministry of Health, Labour and Welfare)\*  
Mr. Hajime Kojima (Section for the Evaluation of Novel Methods, Division of Pharmacology, BSRC, NIHS): Secretary

\* Arrival at post day: 1st August 2013





*In vitro* 皮膚透過試驗

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*In vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) の  
評価会議報告書

JaCVAM 評価会議

平成 25 年 10 月 21 日

## JaCVAM 評価会議

- 吉田武美 (日本毒性学会) : 座長  
浅野哲秀 (日本環境変異原学会)  
五十嵐良明 (国立医薬品食品衛生研究所 生活衛生化学部)  
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任期 : 平成 24 年 4 月 1 日～平成 26 年 3 月 31 日

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以上

当該試験法は、以下の通知やガイドラインに規定されるヒトまたは実験動物から摘出した皮膚を用いた化学物質の皮膚透過性を測定する試験法である。対応する従来試験は、ヒトまたは実験動物の皮膚に化学物質を直接塗布又は貼付してその血中濃度を測定する試験であるが、特定のプロトコルを規定した試験法ではない。

一方、ラットの皮膚に放射性ラベルした化学物質を直接塗布又は貼付して血中、組織、尿中の放射活性を測定する試験法は OECD TG 427 (Skin Absorption: In Vivo Method)として規定されているが、化学物質の代謝物も含んだ測定であるため、当該試験法に対応する従来試験法には該当しない。

- ① Opinion on Basic Criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients - updated March 2006 (Adopted by the SCCP during the 7<sup>th</sup> plenary of 28 March 2006)
- ② Test guideline 428: OECD Guideline for the Testing of chemicals: Skin Absorption: *in vitro* Method (Adopted 13 April, 2004)
- ③ COLIPA GUIDELINES: Guidelines for percutaneous Absorption/Penetration (Edition of 1997)

今回、異なる機関から示されている上記の皮膚透過性試験法について「皮膚透過試験評価委員会」がとりまとめた報告<sup>1)</sup>、<sup>2)</sup>を受け、以下の10項目について評価したので報告する。

#### <審議内容>

1. 当該試験法は、どのような従来試験法を代替するものか。または、どのような毒性を評価あるいは予測するものか。

当該試験法は、ヒトまたは実験動物の皮膚に化学物質を直接塗布又は貼付して全身曝露量を把握する従来の *in vivo* による（皮膚からの吸収量を測定する）方法を代替するものである。

当該試験法は化学物質の皮膚を介する透過性を測定するものであり、毒性を評価あるいは予測するものではない。

2. 当該試験法と従来試験法の間にはどのような科学的なつながりがあるか？

当該試験法はヒトまたは実験動物から摘出した皮膚を用いて化学物質の皮膚透過量を評価する *in vitro* のモデル系であり、皮膚透過量から全身曝露量の推定が可能である。

従来の試験はヒトまたは実験動物の皮膚に化学物質を塗布または貼付し、吸収された化学物質の量(血中濃度)を測定する *in vivo* 試験である。

両試験ともに、皮膚からの化学物質の吸収に対する主要なバリアとなる角質層を有する点で科学的なつながりがある。

3. 当該試験法とそのデータは、透明で独立な科学的評価を受けているか。

当該試験法は、それぞれ、EU、OECD および欧州化粧品協会から発行されたもので、データは透明性があり独立した評価が行われている。

検討対象の3試験法に関する公式の検証はなされていないが、OECDの専門家はOECD TG 428を支持するに十分なデータのあることに合意している<sup>3)</sup>。

4. 当該試験法は、従来試験法の代替法として、どのような物質又は製品を評価することを目的としているか。

従来試験法は皮膚に適用又は曝露した化学物質の血中濃度-時間曲線下面積(AUC)を求める方法であるのに対し、当該試験法は皮膚適用又は曝露した化学物質の皮膚を介する吸収量を求める試験である。

当該試験法は、一般化学物質の皮膚透過性を把握するほか、化粧品、皮膚適用の外用医薬品(貼付剤)および医薬部外品等、又はそれら製品に含まれる新規原料物質のために、経皮的全身曝露を評価する従来試験の代替法として利用が可能である。

5. 当該試験法は、ハザード評価あるいはリスク評価のどちらに有用であるか。

当該試験法は、本試験結果から得られる化学物質の皮膚透過量に基づき、*in vivo*での経皮投与による全身曝露量を推定することが可能であるので、当該化学物質のリスク評価に有用である。

6. 当該試験法は、目的とする物質又は製品の毒性を評価できるか。その場合、当該試験法の適用条件が明確になっているか。

当該試験法は、対象とする化学物質又は製品の全身曝露による吸収量を推定評価するもので、毒性評価を直接目的とするものではない。

なお、対象とする化学物質の物性に応じた当該試験法の適用条件等は明確になっている。

7. 当該試験法はプロトコルの微細な変更に対して十分頑健であるか。

プロトコルの微細な変更には頑健であるが、試験に用いる摘出皮膚の部位や性状に応じて、結果の解釈を慎重に行う必要がある(新鮮皮膚か凍結皮膚か、毛穴の有無他)。

8. 当該試験法の技術習得は、適切な訓練と経験を経ている担当者にとって容易なものであるか。試験法の実施に特殊な設備が必要か。

当該試験に用いる摘出皮膚は、酵素や熱および化学処理等により剥離した表皮、あるいは厚さ200~400 μmに薄切した皮膚組織である。市販の標品を用いる限り、その後の培養や試験手技等の技術習得は容易なものであるが、ダーマトームで皮膚を薄切して試験標品を自ら作製する技術は熟練を要する。

また、皮膚標品を自ら作製する場合にはダーマトームが必要となる。トリチウム水を用いた皮膚バリアの健全性の確認(Integrity測定)および標識された被験物質を用いる場合にはRI設備が必要となる。

9. 当該試験法は、従来試験法と比べて時間的経費的に優れているか。

当該試験法では、動物実験に伴う経費が削減でき、経費的に優れている。ただし、RIを使用する場合は経費的に劣る。

一方、時間的な優位性についての検討はなされていない。

10. 当該試験法は、動物福祉の観点及び科学的見地から、目的とする物質又は製品の毒性を評価する代替法として、行政上利用することは可能か。

当該試験法は動物福祉の3Rの観点から推奨され、欧米では一般的に使われ、我が国でも経皮吸収試験に広く用いられている。当該試験法は、化学物質の皮膚からの透過性を測定するものであり、毒性を評価あるいは予測するものではないが、目的とする物質又は製品の全身曝露量を推定する代替試験法として、行政上利用することは可能である。

参考文献

- 1) 「*In vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験)」 皮膚透過試験評価委員会編
- 2) 「*In vitro* 皮膚透過性試験法の確立の経緯」 皮膚透過試験評価委員会編
- 3) OECD (2000). Report of the Meeting of the OECD Extended Steering Committee for Percutaneous Absorption Testing, Annex 1 to ENV/JM/TG(2000)5. OECD, Paris.





*In vitro* 経皮吸収試験（*In vitro* 皮膚透過試験）評価報告書

平成 22 年 9 月 11 日

皮膚透過試験評価委員会

委員名

杉林堅次（城西大学）

藤井まき子（昭和薬科大学）

藤堂浩明（城西大学）

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## 1. 目的

化粧品や医薬部外品の安全性評価のために用いられている *in vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) に関する各種ガイドラインを比較し、信頼性や長短所等について議論し、採否の可否を検討する。

## 2. 検討した資料の名称とその理由

- ・ SCCP opinion on basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients (2006) <sup>1)</sup>.
- ・ OECD guideline for the testing of chemicals, TG428, skin absorption: *In vitro* method (2004) <sup>2)</sup>.
- ・ Colipa regulatory, Guidelines for percutaneous absorption/penetration (1997) <sup>3)</sup>.
- ・ WHO, Environmental Health Criteria 235, Dermal Absorption (2006) <sup>4)</sup>.

いずれの評価法もガイドライン、ガイダンスとして公知のものであり、汎用性も高いために検討した。

## 3. 試験法の概要 (手順など)

別紙 1 参照

## 4. 試験法として採用の可否

いずれの試験も科学的、倫理的にも評価するには十分である。但し、各試験の適用限界と実施する上での問題点を考慮する必要がある。

### 4-1 *In vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) の適用限界とガイドラインにそって実施する上での問題点

#### 4-1-1 経皮吸収試験結果の具体的な利用法の記載

化粧品原料の経皮吸収試験の目的は、実使用条件においてヒト全身循環系に入る可能性がある被験物質の質的および (あるいは) 量的な情報を得ることである。特に後者は、個々の物質についての反復投与毒性試験の無毒性量 (NOAEL) とともに、安全係数 (MOS、margin of safety) を算出する上で必要である (SCCP/0970/06) <sup>1)</sup>。

OECD TG 428<sup>2)</sup>は記載なし。

化粧品成分の試験および安全性評価に関する SCCP ガイダンス第 6 版 <sup>5)</sup>に記載されている安全係数の求め方と注意点を表 1 に示す。

表1 安全係数 (Margin of Safety: MOS) <sup>5)</sup>

化粧品成分の安全性評価の最終段階であるリスクの評価では、不確実性の要因が適用される。化粧品の場合、この要因は安全係数 (MOS) と呼ばれる。一般に、MOS は最小無影響量 (無毒性量) (NO(A)EL) を、想定される化粧品成分の全身暴露量 (Systemic Exposure Dosage: SED) で除すことによって

$$\text{MOS} = \frac{\text{NO(A)EL}}{\text{SED}}$$

として計算できる。MOS 値は試験動物群から平均的なヒトへ、次に平均的なヒトから感受性の高いグループへと外挿するのに用いられる。一般に、ある物質が安全に使用できると宣言するためには MOS は 100 以上でなければならないとされている。

SED の計算は、予想される最高濃度に基づく一定時間の生物学的利用能の絶対量 ( $\mu\text{g}/\text{cm}^2$ ) に基づくことが望ましいが、経皮吸収率に基づいて行うこともできる。後者の場合、得られる数値は皮膚に適用される用量に依存する。このとき、評価する濃度が低いほど経皮吸収率は高くなる可能性があることから、予想される最低濃度を含まなければならない。

OECD ガイドライン 428 <sup>7)</sup> (経皮吸収: *in vitro* 法) に従い、*in vitro* 試験では、固体で通常 1~5  $\text{mg}/\text{cm}^2$ 、液体で最高 10  $\mu\text{L}/\text{cm}^2$  というヒトへの暴露をシミュレートした適用量を使用しなければならない。

試験物質の適用量が 2  $\text{mg}/\text{cm}^2$  未満の *in vitro* 試験を実施することは技術的に不可能であるが、通常皮膚に適用される化粧品量は実使用条件下で 1  $\text{mg}/\text{cm}^2$  に満たないことが経験から示されている。したがって、*in vitro* 試験では実使用条件を上回る量が適用され、試験用量の経皮吸収率%を SED の計算に使用すると、全身暴露量が過小評価されることになる。したがって、経皮吸収をパーセンテージで表す場合には、*in vitro* 試験から得られた吸収量も実使用条件下で適用した用量のパーセンテージで表す必要がある。これは実使用条件下で適用する製剤の既定量と、表に示す製品種類の皮膚表面積 (SSA) の既定値 (6-2 項参照) の比によって推定することができる。

以上のように、化学物質の経皮吸収がどのように記載報告されているかによって、SED の計算方法には 2 種類あると結論づけられる。

#### 4-1-2 *In vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) の適用限界

*In vivo* 条件下では、微少循環系（血管およびリンパ管）が化合物を皮膚組織から体循環コンパートメントへ運んでいる（吸収、resorption）。*In vitro* の条件下では、そのような吸収過程は評価できない（SCCP/0970/06）<sup>1)</sup>。

表皮は絶えず増殖、分化、落屑を繰り返しており、一日当たり約一層分の角質細胞層が取り除かれている。局所適用した場合、*in vitro* 試験における皮膚上、特に角層や毛嚢脂腺で検出された異物は、*in vivo* においては、それぞれ落屑あるいは皮脂分泌により取り除かれる。これらの過程も *in vitro* では再現できないので、*in vitro* 系での最終的な表皮（角層）中濃度は、*in vivo* レベルと比較して高くなる（SCCP/0970/06）<sup>1)</sup>。

試験化合物が表皮の組織に不可逆的に結合することがあるが、これは *in vivo* においては皮膚表面の落屑によって除去される。この現象が示唆された場合には、別の試験により実証しなければならない（SCCP/0970/06）<sup>1)</sup>。

試験化合物は、拡散セルに装着された皮膚サンプルの上に、適切な製剤を用いて適用されなければならない（SCCP/0970/06）<sup>1)</sup>。単純溶媒系でなく製剤での実施が不可欠である。

#### 4-1-3 *In vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) の問題点

##### 試験の原理

摘出した皮膚を用いた *in vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) の正当性は、表皮、特に角層が生体への異物の吸収や取り込みに対する主要なバリアーとなっているという事実に基づいている（SCCP/0970/06）<sup>1)</sup>。

問題点：水溶性化合物の皮膚透過性については角層がバリアー機能となることが知られているが、化粧品素材においては油等の脂溶性化合物がかなりの数存在し、それらの皮膚透過性には角層だけでなくそれ以下の表皮や真皮もまたバリアーとなると考えられる。したがって、角層以下の層のバリアー能の寄与が高い物質の *in vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) には特に注意が必要である。

##### 使用する皮膚

WHO は最適標準 (gold standard) としてヒト皮膚の使用を勧めている<sup>4)</sup>。もちろん、ヒト皮膚が経皮吸収試験に最も適した試料であるが、それらはいつても容易に入手

できるとは限らない。そこで、代わりにヒト皮膚と同様の透過性を示すブタ皮膚が使用されることもある (SCCP/0970/06) <sup>1)</sup>。

ヒト、ブタまたはラット皮膚を用いることができる (COLIPA) <sup>3)</sup>

ヒトあるいは動物の皮膚を用いることができる (OECD TG 428) <sup>2)</sup>。

被験物質が、*in vivo* 試験においてかなり皮膚代謝を受ける場合には、さらに検討が必要である。凍結皮膚を用いる場合には、代謝能が欠損している可能性があるため、被験物質の代謝が起こらないことや、あるいは代謝物の構造およびその経皮吸収性が正確にはわからないことにも注意しなければならない。したがって、凍結皮膚を用いた *in vitro* 実験では、皮膚中で代謝を受ける化合物の経皮吸収や、その代謝物についても正確な情報を与えないと考えられる (SCCP/0970/06) <sup>1)</sup>。

試験化合物の皮膚代謝が重要である場合には、新鮮な皮膚を使用しなければならない (SCCP/0970/06) <sup>1)</sup>。

**問題点**：日本でも最近、ヒト皮膚を安定的に供給できる体制が整いつつあり、試験計画と試験施設の倫理面が明確であれば供給可能となった。しかしながら、その場合も空輸の関係で、凍結皮膚を用いる場合がほとんどである。したがって、日本国内において、新鮮なヒト皮膚を用いて *in vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) を実施し、皮膚代謝まで明らかにすることは非常に難しいと考える。

使用可能な皮膚は、split-thickness (200~500  $\mu\text{m}$ ) または full-thickness (500~1000  $\mu\text{m}$ ) である [Sanco/222/2000]<sup>6)</sup>。ダーマトームで薄切された皮膚も使用することができる。皮膚厚は文献に記載されている適切な方法で測定する。皮膚は実験用セルにあうように用意しなければならない (SCCP/0970/06) <sup>1)</sup>。

試験皮膚には、酵素、熱、化学処理により剥離した表皮、あるいはダーマトーム等で厚さ 200~400  $\mu\text{m}$  に薄切した皮膚を用いる (OECD TG 428) <sup>2)</sup>。

**問題点**：ダーマトームで皮膚を厚さ 200~400  $\mu\text{m}$  に薄切する技術は非常に難しく、かなりの熟練を要する。

表皮シートを使用する場合には、その理由が必要である。表皮膜はもろいことがあり、このモデルではマスバランス手法 (例：テープストリッピング) を適用することができない。表皮膜の使用は、ヒトの *in vivo* 経皮吸収を過大評価する可能性があることも指摘されている (SCCP/0970/06) <sup>1)</sup>。

**問題点**：体毛は真皮側に存在する毛根から皮膚表面に伸びているため、剥離した表皮においては毛穴が貫通している。毛穴を通して化学物質が通過する可能性もあるため、

経皮吸収量を過大に評価する危険性がある。

## 分析

皮膚および/あるいはレシーバー溶液のサンプルは、液体シンチレーションカウンター、HPLC、GC、あるいは他の適当な方法で、適切にそしてバリデートされた方法を用いて、分析されなければならない。分析方法の感度、再現性、精度が証明されなければならない (SCCP/0970/06) 1)。

問題点 : Cold 分析を実施する場合には、分析方法のバリデーションが要求される。OECD ガイドラインには、バリデーションに関する記載は存在しない。バリデーションに関しては、FDA のガイドラインが存在し、通常、これに準拠して実施される。FDA のガイドラインの項目は以下の通りである。

### FDA のガイドライン

特異性(ブランク試料、n=6)

真度(3 濃度、n=5)

精度(3 濃度、n=5)

回収率(3 濃度)

検量線 (同じマトリクス)

定量下限

濃度レスポンス

安定性

凍結融解安定性 (3 回の凍結融解、

低濃度と高濃度で各 n=3)

短期室温安定性 (低濃度と高濃度で各 n=3)

長期保存安定性 (低濃度と高濃度で各 n=3)

保存溶液安定性

測定実測試料中安定性

バリデーションの問題点として、OECD あるいは SCCP に項目の記載がないことから、FDA のガイドラインに準拠することになるが、その場合、上記の全項目を実施する事になり、金額面で、実施困難になる場合がある。

## レセプター溶液

レセプター液の組成は、被験物質の拡散を妨げないように選択する。例えば、試験条件下の被験物質のレセプター液中での溶解性および安定性は保証されなければならない。生理食塩液や等張緩衝液が一般的に親水性化合物に用いられる。親油性物質に対しては、血清アルブミンまたは適当な可溶化剤・乳化剤を添加することができるが、その際皮膚状態 (membrane integrity) を悪化させてはならない。レセプター液は分



析操作にも妨害しないようにするべきである (SCCP/0970/06) <sup>1)</sup>。

**問題点**：化粧品素材においては、油等の脂溶性化合物がかなりの数存在し、その場合、生理食塩液では溶解しない。したがって、古くから、*in vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) の報告は、多くが水溶性化合物に関するものである。一方、脂溶性化合物の報告も存在するが、その場合、レセプター溶液に、可溶化剤/乳化剤として、ポリオキシエチレン(20)オレイルエーテルが用いられる場合が多い<sup>4)</sup>。しかしながら、この場合にも化合物の脂溶性によっては溶解性に限界がある。試験条件下の被験物質のレセプター液中での溶解性が被験物質の拡散を妨げないように選択すると述べられているが、これは暴露時間終了時の被験物質の透過量が、十分溶解可能な溶媒を選択することである。しかしながら、暴露時間終了時の被験物質の透過量は試験を実施しないとわからないので、実際に試験を実施して、被験物質の拡散を妨げない溶媒であるか否かを判断することは難しい。したがって、脂溶性化合物の *in vitro* 経皮吸収試験 (*in vitro* 皮膚透過試験) に用いることができるレセプター溶液のいくつかの記載が必要と考える。

原則としてレセプター液は、生理的 pH 値にすべきである。これに逸脱する場合には正当化する必要がある。例えば、WHO 235<sup>4)</sup>で提案されている 50/50 エタノール/水 を用いる場合には、それが皮膚 integrity には影響を及ぼさないことを示し、毒性的関係書類に記載しなければならない。早期の採取時間で連続的な ND 値とならないようにするため、レセプター液量は最小限にしなければならない。

**問題点**：エタノールが 5%以上含まれると、経皮吸収性に影響を及ぼすことが知られている。WHO 235<sup>4)</sup>で提案されている 50/50 エタノール/水は使用できないと考える。

レセプター液には、実験中の気泡の発生を抑えるため脱気した溶液を用いる。また、試験期間中、非流出型拡散セルでは十分に攪拌し、流出型拡散セルでは絶えず液を流しておかなくてはならない。

**問題点**：レセプター溶液に、生理食塩液を用いる場合は、脱気することは可能であるが、脂溶性化合物で、レセプター溶液に可溶化剤/乳化剤を添加する場合は、脱気すると泡立つため、脱気することは非常に難しい。

レセプター溶液は、分析手法を妨げてはいけない。試験システムの選択は研究報告書で正当化されるべきである。また、レセプター溶液に透過した化合物の量は、吸収の過小評価を引き起こさないために、飽和レベルの 10%を超えるべきではない。

問題点：被験物質が油分の様な場合は、可溶化剤/乳化剤を用いても、その溶解性には限界がある。レセプター溶液に透過した化合物の量が飽和レベルの 10%以下となると、油分の経皮吸収試験を実施することは非常に困難である。

### 皮膚の integrity

皮膚バリアーのチェック (=integrity) は試験に必須である。これは、指標となる化合物（例：トリチウム水、カフェイン、スクロース）の皮膚透過性を測定するか、もしくは物理的方法（例：TEWL(Transepidermal Water Loss)、TER(Transcutaneous Electrical Resistance)）によって確認する。また、得られたデータは試験報告書に記載しなければならない。

問題点：皮膚の integrity をチェックする方法としてトリチウム水を用いる場合が多い。日本の放射線障害予防規定においては、現在、国際基準に合わせる意味で、トリチウムは濃度で 1MBq/g、数量で 1GBq を下限値として、Radio Isotope (RI) としての取り扱いを免除することになっている。しかしながら、歴史的な背景から、日本の放射能に対する認識は欧米諸国とは異なる。したがって、本規定が運用されることはほとんどなく、トリチウムを用いる場合は、施設の規定で、やはり、RI 実験となり、RI 施設で行うことが義務づけられる。また、トリチウム水で皮膚の integrity をチェックした皮膚は、RI 施設から持ち出すことはできないことから、試験物質の透過性も RI 施設内で実施することになる。試験物質に放射性標識体を用いる場合は問題ないが、Cold の機器分析を実施する場合は、RI 室に分析機器を常設する必要がある。分析機器によっては、LC/MS/MS、ICP/MS 等、かなり高価なものがあるため、必ずしも RI 施設内に設置することが難しい現状である。これを避ける方法として、皮膚の integrity はトリチウム水でチェックし、その皮膚とは別に、その近傍の皮膚を用いて、RI 施設外で試験化合物の試験を行い、Cold で機器分析を実施する方法がある。

しかしながら、この様な方法は、ガイドラインに記載されておらず、その正当性に関しては不明である。OECD TG428<sup>2)</sup>ではトリチウム水の使用を推奨している。

## 5. 総括

動物実験の 3 Rs (Replacement、Refinement、Reduction) の普及を受け<sup>8,9)</sup>、欧米では化粧品規制や REACH に対応するため、動物実験代替法の利用が推奨されている。よって、欧米では安全性評価における経皮吸収試験や皮膚透過性は *in vitro* 試験が中心である<sup>10)</sup>。

本委員会においては各種、*in vitro* 試験法についての長短所を議論してきたが、いず

れの試験も適用限界を理解し、実施する上での問題点を考慮しながら実施する必要がある。但し、適用限界を理解した上で、ガイドラインやガイダンスに準拠した正しい試験で行った *in vitro* 試験から *in vivo* の結果を推定することが可能であると思われる。正しい方法というのは皮膚の選定、レセプター相の選定、皮膚代謝の有無、試験物質の物性等を十分に考慮することである。

本検討会で評価した 4 資料はいずれも公知で、欧米では一般的に用いられている試験法であることを鑑みると、適用限界を十分に理解した上で評価すれば、化学物質のリスク評価および効能評価の一つの評価系として十分に活用できる。

#### 参考文献

- 1) SCCP/0970/06 Opinion on basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients (2006)
- 2) OECD428 Guideline for the testing of chemicals, skin absorption: *In vitro* method (2004)
- 3) Colipa regulatory, Guidelines for i percutaneous absorption/penetration (1997)
- 4) WHO, Environmental Health Criteria 235, Dermal Absorption (2006)
- 5) SCCP The sccp's notes of guidance for the testing of cosmetic ingredients and their safety evaluation 6th revision
- 6) Sanco/222/2000 rev. 7, Guidance document on dermal absorption (2004)
- 7) Food and Drug Administration, Guidance for industry: Bioanalytical method validation (2001).
- 8)動物の愛護及び管理に関する法律  
[http://www.env.go.jp/nature/dobutsu/aigo/amend\\_law2/law.pdf](http://www.env.go.jp/nature/dobutsu/aigo/amend_law2/law.pdf)
- 9)実験動物の飼養及び保管並びに苦痛軽減に関する基準  
[http://www.env.go.jp/nature/dobutsu/aigo/law\\_series/nt\\_h180428\\_88.html](http://www.env.go.jp/nature/dobutsu/aigo/law_series/nt_h180428_88.html)
- 10)Commission Staff Working Documents(2004)Time Tables for the phasing-out of animal testing in the framework of the 7<sup>th</sup> Amendment to the Cosmetics Directive (Council Directive 76/768/EEC); EN, SEC82004,1210

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## JaCVAM 評価会議からの質問に対する回答

杉林堅次、藤堂浩明（城西大）、藤井まきこ（昭和薬科大）

### 1) 「皮膚透過性、経皮吸収、皮膚吸収などの言葉の定義」

一般に、「皮膚透過性」は単に皮膚を透過する量、速度、およびその性質をいいます。

「*In vitro* 皮膚透過性」などと使います。

一方、「吸収」とは全身循環系（おもに血管系）に化学物質が入ることを意味しますので、「経皮吸収性」は皮膚を経て全身循環系に入る量、速度、およびその性質をいいます。これは *in vivo* に対応する言葉と理解するのが適当です。ただ、世の中には適当に使っている研究者の方が多いと思います。私は皮膚吸収という用語は使いません。どっちかといえば、経皮吸収と同意語かと思います。

杉林先生がコスメテックジャパンに連載している「シリーズ 経皮吸収について考える」その 8 で「経皮吸収」、「皮膚透過」、「皮膚浸透」の定義について、文献を引用し示しています（添付します）。

### 2) 「*in vitro* 皮膚透過性試験はどの試験法の代替法でしょうか。」

*in vivo* 経皮吸収試験（TG427）の代替法と考えています。

### 3) 「AUC の算定式で、「全身クリアランスが既知なら」とありますが、既知である場合はどれくらいあるのでしょうか。」

物質の静脈内投与後の血中濃度-時間曲線のデータがあれば、既知です。医薬品や部外品で静注製剤があれば既知だと思います。効能を発揮する機能性化粧品の有効物質もわかっているケースの方が多いように思います。全身クリアランスについてです。部外品等で使われている有効成分は申請時に明らかにされていると思いますが、その物質のクリアランスが測定されているかという、されていないほうが多いのではないかと思います。しかし、物質が同じであれば、AUC と吸収量は比例するので、間接的に吸収量を類推していた AUC 法に代わり、吸収量を把握する方法を採用することに問題はないと考えます。

### 4) 「皮膚透過性の結果から、経皮全身曝露が推定できるはずがない。」

皮膚透過性試験は、化粧品・医薬部外品等における皮膚透過量を把握し、安全係数を求めるための方法である。あるいは、皮膚透過性がほとんどないことを証明する試験法に過ぎないとの意見について」

皮膚透過した化学物質のほとんど（通常 95%以上）は全身循環系に行きます。皮膚透過試験から経皮全身暴露が推定できるはずがないといいきる科学者が世の中にいること自体驚きです。東大生産研の酒井先生などは、私の意見をよく理解してくれると思います。一般に、薬学の「薬剤学」「製剤学」の分野の先生、化学工学の先生などはよく理解しています。薬剤師 国家試験にも出る内容であるかと思っています。なお、*in vitro* 皮膚透過性から血中濃度を予測する計算ソフトも市販されています（九州工業大学 東条先生の SkinCad が有名です。ちなみに彼も化学工学出身です）。製薬企業の研究者の多くも理解すると思います。一方、化粧品研究者はまだ情報科学（インフォマティクス）や数理計算を利用している研究者が少ないのが現状です。代替法研究でも、*in silico* や前述した酒井先生の方法論はよく理解されていません。

ラットの *in vitro* 皮膚透過速度と *in vivo* 経皮吸収速度は、単純な基剤系で比較したことがあります、ほぼ一緒です。違う場合の理由を探す方が難しいくらいです。

もちろん、皮膚中の代謝やトランスポーター介在の皮膚透過も見られる可能性があるのも、正しく *in vitro* 皮膚透過実験をすることが重要であることは言うまでもありません。

物質収支は *in vitro* も *in vivo* もほとんど同じです。ここでは、皮膚を通る速度しか比較していないのですから。東大から理研に移った杉山先生が東大教授になられたころは PK 研究をされていました。臓器レベルで物質収支を研究した結果、臓器や体の生理学的薬物速度論モデルを組み立てられました。私の方法論もそれらと、ほとんど変わりません。

以上

## 別紙 1

### 3.試験法の概要（手順など）

表1-1. ガイドラインの比較 (in vitro 経皮吸収試験)

Guideline		SCCP (2006.3)	OECD (TG428, 2004.4)	Colipa (1999)
拡散セル		Flow through/Static cell (選択は化合物次第)	Flow through/Static cell (有限系：開放, 無限系：閉塞)	チャンバー(ドナー, レセプター)で皮膚を挟んだもの. セルの選択は薬剤による.
レセプター液		親水性： 生食, 緩衝生食液 脂溶性： アルブミン, 可溶化剤/乳化剤の添加は可	<ul style="list-style-type: none"> <li>対象物質が溶解すること</li> <li>皮膚に影響を与えないこと</li> <li>代謝試験では皮膚能を維持すること</li> </ul>	親水性： 生食液, 緩衝生食液 脂溶性： アルブミン, 可溶化剤を含む液
皮膚膜	種	○ ヒト/ブタ, × げっ歯類 (△ 培養皮膚/再構成皮膚)	ヒト/動物 (ヒトの場合は倫理下で)	記載なし (Std protocol ではヒト, ブタ, ラット)
	部位	ヒト： 腹, 脚, 胸囲 ブタ： 腹, 胸, 背, 側, 耳	記載なし	記載なし
	皮膚厚	ヒト： ○ 200-500 μm △ 500-1,000 μm (表皮膜は過大評価の恐れ) ブタ： 500-1,000 μm でもOK	○ 表皮膜 (酵素/熱処理) /剥離皮膚 (200-400 μm) △ full-thickness skin × >1mm	○ whole, split-thickness ( < 1 mm とすること)
Integrity test		必須： <sup>3</sup> H <sub>2</sub> O, カフェイン, ショ糖, TER, TEWL	必須： 方法については記載なし	必須： <sup>3</sup> H <sub>2</sub> O, TEWL など
被験物質		RI 体は大規模ロット (Cold体) とは若干異なる特徴を示す	RI ラベル化が理想的標準処方で1 濃度以上	-
適用量		固形/半固形： 2-5 mg/cm <sup>2</sup> 液体： ~10 μL/cm <sup>2</sup>	固形： 1-5 mg/cm <sup>2</sup> 液体： ~10 μL/cm <sup>2</sup>	実使用で (consumer use)
n 数(/1サンプル)		> 6 (3ドナー× 2 以上)	最低4	-

表1-2. ガイドラインの比較 (in vitro 経皮吸収試験)

Guideline	SCCP (2006.3)	OECD (TG428, 2004.4)	Colipa (1999)
皮膚表面温度	32±1 °C	32±1 °C (湿度は 30-70 %)	30-32 °C
適用時間	24 時間 (越える場合は皮膚状態に注意)	<ul style="list-style-type: none"> <li>・ 一般的には 24 時間 (対象物質の透過性による)</li> <li>・ 適用途中でサプリングを実施 (透過プロファイルを図示する)</li> </ul>	24 時間
測定部位	<ul style="list-style-type: none"> <li>・ 皮膚表面</li> <li>・ 角層</li> <li>・ 表皮 (角層を除く)</li> <li>・ 真皮</li> <li>・ レセプター液</li> </ul>	<ul style="list-style-type: none"> <li>・ ドナーチャンバー</li> <li>・ 皮膚表面</li> <li>・ 皮膚</li> <li>・ レセプター液</li> </ul> <p>場合によっては、塗布部位、塗布外部 (セル密着部位), 角層, 表皮, 真皮に分ける.</p>	<ul style="list-style-type: none"> <li>・ 皮膚表面</li> <li>・ 角層</li> <li>・ 表皮 (角層を除く)</li> <li>・ 真皮</li> <li>・ レセプター液</li> </ul>
回収率	100±15 % (逸脱なら調査/説明)	RI 使用時の目標は, 100±10 % (逸脱なら理由を記載)	100±15 %
算出値	絶対量 (µg/cm <sup>2</sup> ) 吸収率 (% of dose)	<p>有限系の場合 :</p> <p>皮膚表面量, 皮膚内量, レセプター液の速度および量あるいはパーセンテージ.</p> <p>無限系の場合 :</p> <p>透過係数を算出. パーセンテージは必要ない.</p>	絶対量 (µg/cm <sup>2</sup> ) 吸収率 (% of dose)



## OECD GUIDELINE FOR THE TESTING OF CHEMICALS

### Skin Absorption: *in vitro* Method

#### INTRODUCTION

1. This test guideline has been designed to provide information on absorption of a test substance applied to excised skin. It can either be combined with the OECD Test Guideline for Skin Absorption: *In vivo* Method (1), or be conducted separately. It is recommended that the OECD Guidance Document for the Conduct of Skin Absorption Studies (2) be consulted to assist in the design of studies based on this Test Guideline. The OECD Guidance Document has been prepared to facilitate the selection of appropriate *in vitro* procedures for use in specific circumstances, to ensure the reliability of results obtained by this method.

#### INITIAL CONSIDERATIONS

2. The methods for measuring skin absorption and dermal delivery can be divided into two categories: *in vivo* and *in vitro*. *In vivo* methods on skin absorption are well established and provide pharmacokinetic information in a range of animal species. An *in vivo* method is separately described in another OECD guideline (1). *In vitro* methods have also been used for many years to measure skin absorption. Although formal validation studies of the *in vitro* methods covered by this Test Guideline have not been performed, OECD experts agreed in 1999 that there was sufficient data evaluated to support the *in vitro* Test Guideline (3). Further details that substantiate this support, including a significant number of direct comparisons of *in vitro* and *in vivo* methods, are provided with the Guidance Document (2). There are a number of monographs that review this topic and provide detailed background on the use of an *in vitro* method (4)(5)(6)(7)(8)(9)(10)(11)(12). *In vitro* methods measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise non-viable skin to measure diffusion only, or fresh, metabolically active skin to simultaneously measure diffusion and skin metabolism. Such methods have found particular use as a screen for comparing delivery of chemicals into and through skin from different formulations and can also provide useful models for the assessment of percutaneous absorption in humans.

3. The *in vitro* method may not be applicable for all situations and classes of chemicals. It may be possible to use the *in vitro* test method for an initial qualitative evaluation of skin penetration. In certain cases, it may be necessary to follow this up with *in vivo* data. The Guidance Document (2) should be consulted for further elaboration of situations where the *in vitro* method would be suitable. Additional detailed information to support the decision is provided in an OECD Expert Meeting report (3).

4. This guideline presents general principles for measuring dermal absorption and delivery of a test substance using excised skin. Skin from many mammalian species, including humans, can be used. The permeability properties of skin are maintained after excision from the body because the principal diffusion barrier is the non-viable *stratum corneum*; active transport of chemicals through the skin has not been identified. The skin has been shown to have the capability to metabolise some chemicals during percutaneous absorption (6), but this process is not rate limiting in terms of actual absorbed dose, although it may affect the nature of the material entering the bloodstream.

### **PRINCIPLE OF THE TEST**

5. The test substance, which may be radiolabelled, is applied to the surface of a skin sample separating the two chambers of a diffusion cell. The chemical remains on the skin for a specified time under specified conditions, before removal by an appropriate cleansing procedure. The receptor fluid is sampled at time points throughout the experiment and analysed for the test chemical and/or metabolites.

6. When metabolically active systems are used, metabolites of the test chemical may be analysed by appropriate methods. At the end of the experiment the distribution of the test chemical and its metabolites are quantified, when appropriate.

7. Using appropriate conditions, which are described in this guideline and accompanying guidance document (2), absorption of a test substance during a given time period is measured by analysis of the receptor fluid and the treated skin. The test substance remaining in the skin should be considered as absorbed unless it can be demonstrated that absorption can be determined from receptor fluid values alone. Analysis of the other components (material washed off the skin and remaining within the skin layers) allows for further data evaluation, including total test substance disposition and percentage recovery.

8. To demonstrate the performance and reliability of the test system in the performing laboratory, the results for relevant reference chemicals should be available and in agreement with published literature for the method used. This requirement could be met by testing an appropriate reference substance (preferably of a lipophilicity close to the test substance) concurrently with the test substance or by providing adequate historical data for a number of reference substances of different lipophilicity (e.g. caffeine, benzoic acid, and testosterone).

### **DESCRIPTION OF THE METHOD**

#### **Diffusion cell**

9. A diffusion cell consists of a donor chamber and a receptor chamber between which the skin is positioned (an example of a typical design is provided in Figure 1). The cell should provide a good seal around the skin, enable easy sampling and good mixing of the receptor solution in contact with the underside of the skin, and good temperature control of the cell and its contents. Static and flow-through diffusion cells are both acceptable. Normally, donor chambers are left unoccluded during exposure to a finite dose of a test preparation. However, for infinite applications and certain scenarios for finite doses, the donor chambers may be occluded.

#### **Receptor fluid**

10. The use of a physiologically conducive receptor fluid is preferred although others may also be used provided that they are justified. The precise composition of the receptor fluid should be provided. Adequate solubility of the test chemical in the receptor fluid should be demonstrated so that it does not act as a barrier to absorption. In addition, the receptor fluid should not affect skin preparation integrity. In a flow-through system, the rate of flow must not hinder diffusion of a test substance into the receptor fluid. In a static cell system, the fluid should be continuously stirred and sampled regularly. If metabolism is being studied, the receptor fluid must support skin viability throughout the experiment.

### **Skin preparations**

11. Skin from human or animal sources can be used. It is recognised that the use of human skin is subject to national and international ethical considerations and conditions. Although viable skin is preferred, non-viable skin can also be used provided that the integrity of the skin can be demonstrated. Either epidermal membranes (enzymically, heat or chemically separated) or split thickness skin (typically 200-400  $\mu\text{m}$  thick) prepared with a dermatome, are acceptable. Full thickness skin may be used but excessive thickness (*ca.* > 1 mm) should be avoided unless specifically required for determination of the test chemical in layers of the skin. The selection of species, anatomical site and preparative technique must be justified. Acceptable data from a minimum of four replicates per test preparation are required.

### **Skin preparation integrity**

12. It is essential that the skin is properly prepared. Inappropriate handling may result in damage to the *stratum corneum*, hence the integrity of the prepared skin must be checked. When skin metabolism is being investigated, freshly excised skin should be used as soon as possible, and under conditions known to support metabolic activity. As a general guidance, freshly excised skin should be used within 24 hrs, but the acceptable storage period may vary depending on the enzyme system involved in metabolism and storage temperatures (13). When skin preparations have been stored prior to use, evidence should be presented to show that barrier function is maintained.

### **Test substance**

13. The test substance is the entity whose penetration characteristics are to be studied. Ideally, the test substance should be radiolabelled.

### **Test preparation**

14. The test substance preparation (e.g., neat, diluted or formulated material containing the test substance which is applied to the skin) should be the same (or a realistic surrogate) as that to which humans or other potential target species may be exposed. Any variation from the 'in-use' preparation must be justified.

### **Test substances concentrations and formulations**

15. Normally more than one concentration of the test substance is used in typical formulations, spanning the realistic range of potential human exposures. Likewise, testing a range of typical formulations should be considered.

### **Application to the skin**

16. Under normal conditions of human exposure to chemicals, finite doses are usually encountered. Therefore, an application that mimics human exposure, normally 1-5  $\text{mg}/\text{cm}^2$  of skin for a solid and up to 10  $\mu\text{l}/\text{cm}^2$  for liquids, should be used. The quantity should be justified by the expected use conditions, the study objectives or physical characteristics of the test preparation. For example, applications to the skin surface may be infinite, where large volumes per unit area are applied.

### **Temperature**

17. The passive diffusion of chemicals (and therefore their skin absorption) is affected by temperature. The diffusion chamber and skin should be maintained at a constant temperature close to

normal skin temperature of  $32 \pm 1^\circ\text{C}$ . Different cell designs will require different water bath or heated block temperatures to ensure that the receptor/skin is at its physiological norm. Humidity should preferably be between 30 and 70%.

### **Duration of exposure and sampling**

18. Skin exposure to the test preparation may be for the entire duration of the experiment or for shorter times (i.e., to mimic a specific type of human exposure). The skin should be washed of excess test preparation with a relevant cleansing agent, and the rinses collected for analysis. The removal procedure of the test preparation will depend on the expected use condition, and should be justified. A period of sampling of 24 hours is normally required to allow for adequate characterisation of the absorption profile. Since skin integrity may start to deteriorate beyond 24 hours, sampling times should not normally exceed 24 hours. For test substances that penetrate the skin rapidly this may not be necessary but, for test substances that penetrate slowly, longer times may be required. Sampling frequency of the receptor fluid should allow the absorption profile of the test substance to be presented graphically.

### **Terminal procedures**

19. All components of the test system should be analysed and recovery is to be determined. This includes the donor chamber, the skin surface rinsing, the skin preparation and the receptor fluid/chamber. In some cases, the skin may be fractionated into the exposed area of skin and area of skin under the cell flange, and into *stratum corneum*, epidermis and dermis fractions, for separate analysis.

### **Analysis**

20. In all studies adequate recovery should be achieved (the aim should be a mean of  $100 \pm 10\%$  of the radioactivity and any deviation should be justified). The amount of test substance in the receptor fluid, skin preparation, skin surface washings and apparatus rinse should be analysed, using a suitable technique.

## **DATA AND REPORTING**

### **Data**

21. The analysis of receptor fluid, the distribution of the test substance chemical in the test system and the absorption profile with time, should be presented. When finite dose conditions of exposure are used, the quantity washed from the skin, the quantity associated with the skin (and in the different skin layers if analysed) and the amount present in the receptor fluid (rate, and amount or percentage of applied dose) should be calculated. Skin absorption may sometimes be expressed using receptor fluid data alone. However, when the test substance remains in the skin at the end of the study, it may need to be included in the total amount absorbed (see Guidance Document, paragraph 66). When infinite dose conditions of exposure are used the data may permit the calculation of a permeability constant ( $K_p$ ). Under the latter conditions, the percentage absorbed is not relevant.

**Test report**

22. The test report must include the requirements stipulated in the protocol, including a justification for the test system used and should, comprise the following:

## Test substance:

- physical nature, physicochemical properties (at least molecular weight and log  $P_{ow}$ ), purity (radiochemical purity);
- identification information (e.g. batch number);
- solubility in receptor fluid.

## Test preparation:

- formulation and justification of use;
- homogeneity.

## Test conditions:

- sources and site of skin, method of preparation, storage conditions prior to use, any pre-treatment (cleaning, antibiotic treatments, etc.), skin integrity measurements, metabolic status, justification of use;
- cell design, receptor fluid composition, receptor fluid flow rate or sampling times and procedures;
- details of application of test preparation and quantification of dose applied;
- duration of exposure;
- details of removal of test preparation from the skin, for example, skin rinsing;
- details of analysis of skin and any fractionation techniques employed to demonstrate skin distribution;
- cell and equipment washing procedures;
- assay methods, extraction techniques, limits of detection and analytical method validation.

## Results:

- overall recoveries of the experiment (Applied dose  $\equiv$  Skin washings + Skin + Receptor fluid + Cell washings);
- tabulation of individual cell recoveries in each compartment;
- absorption profile;
- tabulated absorption data (expressed as rate, amount or percentage).

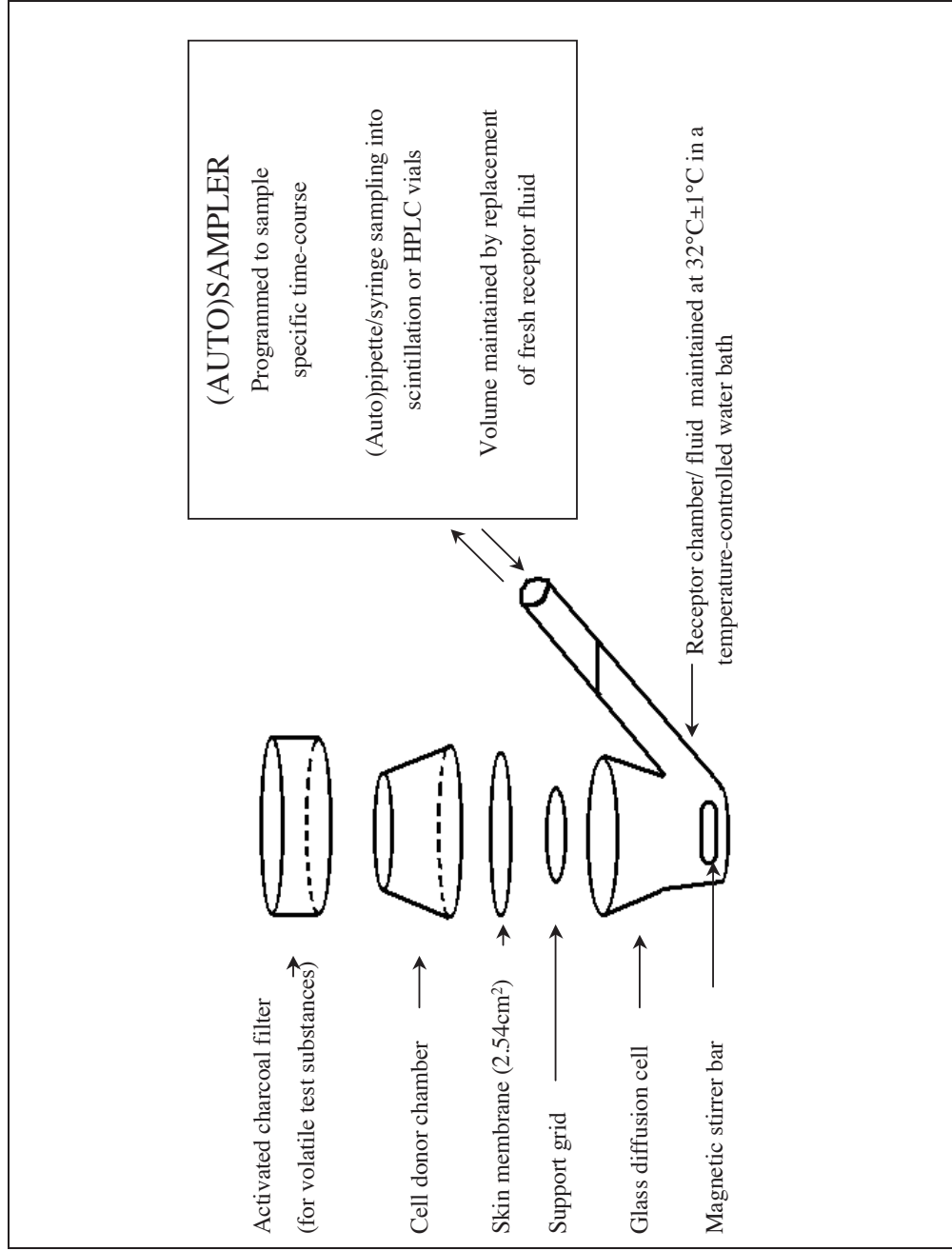
## Discussion of results.

## Conclusions.

**LITERATURE**

- (1) OECD (2004). Test Guideline 427: Skin absorption: *in vivo* Method. OECD, Paris.
- (2) OECD (2004). Guidance Document for the Conduct of Skin Absorption Studies. OECD, Paris.
- (3) OECD (2000). Report of the Meeting of the OECD Extended Steering Committee for Percutaneous Absorption Testing, Annex 1 to ENV/JM/TG(2000)5. OECD, Paris.
- (4) Kemppainen BW and Reifenrath WG. (1990). Methods for skin absorption. CRC Press, Boca Raton.
- (5) Bronaugh RL and Collier, SW. (1991). Protocol for *In vitro* Percutaneous Absorption Studies, in *In vitro Percutaneous Absorption: Principles, Fundamentals and Applications*, RL Bronaugh and HI Maibach, Eds., CRC Press, Boca Raton, pp. 237-241.
- (6) Bronaugh RL and Maibach HI. (1991). *In vitro Percutaneous Absorption: Principles, Fundamentals and Applications*. CRC Press, Boca Raton.
- (7) European Centre for Ecotoxicology and Toxicology of Chemicals (1993). Monograph No. 20, Percutaneous Absorption, ECETOC, Brussels.
- (8) Diembeck W, Beck H, Benech-Kieffer F, Courtellemont P, Dupuis J, Lovell W, Paye M, Spengler J, Steiling W (1999). Test Guidelines for *In Vitro* Assessment of Dermal Absorption and Percutaneous Penetration of Cosmetic Ingredients, *Fd Chem Tox*, 37, 191-205.
- (9) Recommended Protocol for *In vitro* Percutaneous Absorption Rate Studies (1996). US Federal Register, Vol. 61, No. 65.
- (10) Howes D, Guy R, Hadgraft J, Heylings JR *et al.* (1996). Methods for assessing percutaneous absorption. ECVAM Workshop Report ATLA 24, 81 R10.
- (11) Schaefer H and Redelmeier TE. (1996). Skin barrier: principles of percutaneous absorption. Karger, Basel.
- (12) Roberts MS and Walters KA. (1998). Dermal absorption and toxicity assessment. Marcel Dekker, New York.
- (13) Jewell, C., Heylings, JR., Clowes, HM. And Williams, FM. (2000). Percutaneous absorption and metabolism of dinitrochlorobenzene *in vitro*. *Arch Toxicol* 74: 356-365.

Figure 1: An example of a Typical Design of a Static Diffusion Cell for *in vitro* Percutaneous Absorption Studies



ANNEXDEFINITIONS

Unabsorbed dose: represents that washed from the skin surface after exposure and any present on the non-occlusive cover, including any dose shown to volatilise from the skin during exposure.

Absorbed dose: (*in vitro*): mass of test substance reaching the receptor fluid or systemic circulation within a specified period of time.

The absorbable dose: (*in vitro*) represents that present on or in the skin following washing.





EUROPEAN COMMISSION  
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL  
Directorate C - Public Health and Risk Assessment  
C7 - Risk assessment

**SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS  
(SCCP)**

**Opinion on**

**BASIC CRITERIA FOR THE *IN VITRO* ASSESSMENT OF  
DERMAL ABSORPTION OF COSMETIC INGREDIENTS -  
updated March 2006**

Adopted by the SCCP  
during the 7<sup>th</sup> plenary of 28 March 2006

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## 1. BACKGROUND

As early as June 1999, before any OECD guideline on percutaneous absorption was available, important scientific articles on the methodology were published [Beck et al. 1995, Diembeck et al. 1999]. The SCCNFP discussed the scientific progress with external experts in the field and subsequently adopted its first "Basic criteria for the *in vitro* assessment of percutaneous absorption of cosmetic ingredients" [SCCNFP/0167/99]. The publication of such a set of criteria was seen as a pro-active action of the SCCNFP in order to support and speed up the introduction of *in vitro* dermal absorption studies of cosmetic ingredients in the evaluation of dossiers of cosmetic ingredients belonging to Annexes III, IV, VI or VII to Dir. 76/768/EEC. Moreover, the opinion did not only provide basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients, but also discussed some general principles and practical points of the methodology.

In December 2000, two draft OECD Guidelines on dermal absorption became available, namely 1) OECD Draft Guideline 427 on skin absorption: *in vivo* method [OECD 427], and 2) OECD Draft Guideline 428 on skin absorption: *in vitro* method [OECD 428]. The combination of the OECD-publication with gained practical experience, has led to the incorporation of the *in vitro* dermal absorption study in toxicological dossiers of cosmetic ingredients, plant protection products, biocides, etc [98/8/EC, 2000/6/EC, Sanco/222/2000].

Subsequently, in order to provide an up to date guidance for the application of the *in vitro* methodology for cosmetic ingredients, document SCCNFP/0167/99 was revised and updated in 2003 [SCCNFP/0750/03]. Existing documents such as the "Proposed rule for *in vitro* dermal absorption rate testing ..." [US EPA 1999], the "Guidance document on dermal absorption" [Sanco/222/2000], the "Draft Guidance Document for the conduct of skin absorption studies" [OECD 2000], and the "Technical Guidance Document on Risk Assessment part 1" [ECB 2003] were taken into consideration.

The SCCP has noticed that several dossiers have failed to fulfil the requirements as described in document SCCNFP/0750/03. In particular, a recent revision of all hair dye dossiers revealed a number of problems [Pauwels and Rogiers 2004].

The following actions were undertaken:

- The SCCP established a working group, consisting of SCCP members and external experts in the field of dermal absorption. The present opinion results from the work of the expert group, whose task was to reach a consensus concerning a revision of the "SCCP basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients".
- In order to make the SCCP criteria more widely known, it is intended to expand the chapter on dermal absorption in the SCCP Notes of Guidance, 6<sup>th</sup> revision.

## 2. GENERAL PRINCIPLES

### Definitions

The definitions with respect to dermal absorption slightly diverge in different official documents. Hereunder, the definitions as proposed by the WHO [WHO 2005] are given here:

The **percutaneous/dermal absorption** process is a global term which describes the passage of compounds across the skin. This process can be divided into three steps:

- **penetration**, which is the entry of a substance into a particular layer or structure such as the entrance of a compound into the stratum corneum;
- **permeation**, which is the penetration through one layer into another, which is both functionally and structurally different from the first layer;
- **resorption** which is the uptake of a substance into the vascular system (lymph and/or blood vessel), which acts as the central compartment.

The purpose of dermal absorption studies of cosmetic ingredients is to obtain qualitative and/or quantitative information on the amounts that may enter, under in-use conditions, into the systemic compartment of the human body. These quantities can then be taken into consideration to calculate the margin of safety using the NOAEL of an appropriate repeated dose toxicity study with the respective substance.

Justification for the use of *in vitro* dermal absorption studies on isolated skin is based on the fact that the epidermis, in particular the stratum corneum, forms the principal *in vivo* barrier of the skin against penetration and uptake of xenobiotics in the body.

Under *in vivo* conditions, the microcirculatory system (blood and lymph vessels) may carry compounds from the dermis into the central compartment (resorption). *In vitro*, the microcirculation is compromised, thus the potential resorption of a compound cannot be adequately studied in such a setting.

The dermal tissue may retain penetrating compounds that, *in vivo*, would have been removed into the systemic compartment. Thus, either the dermis must be removed prior to *in vitro* investigations (so-called split-thickness skin) or possible *in vitro* retention in the dermis and living epidermis (without stratum corneum) must be taken into account when interpreting the *in vitro* results.

The epidermis renews by continuous outward proliferation, differentiation and desquamation. About one layer of corneocytes is shed off per day. After topical application, xenobiotics detected *in vitro* in the skin, particularly in the stratum corneum and the pilosebaceous units, might *in vivo* have been lost from the skin via desquamation or sebum secretion, respectively. Because these processes are not present *in vitro*, the final epidermal (stratum corneum) levels *in vitro* could be elevated compared with the corresponding *in vivo* levels.

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According to these principles, the following should be included in the protocol for *in vitro* dermal absorption studies:

- i. Studies should be performed on appropriate standardised skin preparations. The respective choice should be justified in the protocol. The WHO recommends human skin as the gold standard.
- ii. At the end of the experiment, a full mass balance should be performed.
- iii. When considerable cutaneous metabolism of the test compound occurs *in vivo*, further studies may be necessary. It should be noticed that frozen skin preparations may lack the enzyme systems for biotransformation of the test compound and may not provide an accurate picture of the formation of metabolites and their dermal absorption. Therefore *in vitro* studies using frozen skin may not provide complete information on the dermal absorption of compounds that undergo biotransformation in the skin nor on their potential metabolite(s) formed. The role of cutaneous biotransformation in the absorption process is still a matter of scientific debate.
- iv. Sometimes an irreversible binding of an ingredient to the epidermis may occur, followed by elimination through *in vivo* desquamation of the skin surface. When this mechanism is assumed, it must be documented by separate experiments.

### 3. PRINCIPLE OF THE TEST

At present only skin preparations of natural origin may be used. Although the quality of cultured or reconstituted skin has improved importantly during the last years [Coquette et al. 2000], these cultures do not possess a complete barrier function comparable to that of living skin.

OECD Guideline 428 should be followed as close as possible, taking into account the guidance given here. Any deviation from the OECD or SCCP guidelines should be documented and justified by appropriate scientific argumentation.

The test substance should be applied in an appropriate formulation on the skin sample which is placed in a diffusion cell (cf. 4.1). The skin is then positioned between the upper and lower chambers of the cell. Diffusion cells may be of static or flow-through design. The integrity of the barrier should be checked by an appropriate method. The test sample should remain in contact with the skin on the donor side for a defined period of time, corresponding to the typical use of the cosmetic end product, such as leave-on or rinse-off conditions. The receptor fluid should be sampled at an early time point (e.g. after 30 minutes), at the end of the experiment and at appropriate time points in between in order to obtain an absorption-time profile. A justification of the procedure used (static or flow-through conditions) should be provided. The skin and/or fluid samples should be analysed by appropriate and validated analytical methods, such as liquid scintillation counting, HPLC, GC or other suitable methods. Information on the sensitivity and repeatability / time-different intermediate precision of the analytical method(s) should be provided.

## 4. FACTORS AFFECTING DERMAL ABSORPTION AND METHODOLOGY

Dermal absorption can be affected by several factors : e.g. physical and chemical properties of the substance, type and composition of the formulation, occlusion, concentration of the substance in the formulation, exposure pattern, skin site of the body and technical aspects of the respective *in vitro* test [Howes et al. 1996, Schaefer and Redelmeier 1996, ECETOC 1993].

In the following section, an overview is given of factors that may affect dermal absorption in *in vitro* dermal absorption studies.

### 4.1. Diffusion cell design

The diffusion cell consists of an upper donor and a lower receptor chamber, separated by the skin preparation under investigation. The stratum corneum faces the donor chamber. Diffusion cells should consist of inert non-adsorbing material. Temperature control of the receptor fluid is crucial throughout the experiment. The skin surface temperature in the diffusion cell should be kept at the *in vivo* skin temperature of  $32 \pm 1^\circ\text{C}$ . Additional dermal absorption studies may be required in some specific cases, e.g. when substance exposure at a higher skin temperature may be expected. The receptor fluid in static cells should be well-stirred throughout the study. The advantage of using a flow-through system is continuous sampling; the advantage of using a static system is the increase in sensitivity for test substances only poorly penetrating the skin.

### 4.2. Receptor fluid

The composition of the receptor fluid is chosen so that it does not limit the extent of diffusion of the test substance, i.e. the solubility and the stability in the receptor fluid of the chemical under investigation have to be guaranteed. Saline or buffered saline solutions are commonly used for hydrophilic compounds. For lipophilic molecules, serum albumin or appropriate solubilisers / emulsifiers are added in amounts which must not interfere with membrane integrity. The fluid should not interfere with the analytical procedure.

As a general rule, the receptor fluid should have a physiological pH. Any deviation from this principle should be justified; e.g. in the case of 50/50 ethanol/water (as proposed in the OECD Guideline), evidence should be included in the dossier, showing that this does not significantly affect the integrity of the skin.

In order to avoid serial non-detects at early sample points, the receptor fluid volume should be kept to a minimum.

The receptor fluid, preferably degassed in order to avoid formation of air bubbles during the experiment, should be thoroughly stirred (static cells) or continuously replaced (flow-through cells) during the entire experiment. The choice of static or flow-through conditions in the receptor cell should be made on a compound-by-compound basis, depending on its theoretical absorption properties and the objective of the study. The choice of the test system should be justified in the study report. The amount of penetrated substance in the receptor fluid should not exceed 10% of its saturation level at any time, in order to minimise interference with the free diffusion process that could produce an underestimation of dermal absorption. The substance should be stable in the receptor fluid for the duration of the *in vitro* test and the subsequent analysis.

### 4.3. Skin preparations

Human skin is obviously the best choice but is not always readily available. Alternatively, pig skin may be used because it shares essential permeation characteristics with human skin. Rat skin is 2 to 10 times more permeable than human skin [Ross et al. 2000] which may result in an overestimation of dermal absorption. Mouse, guinea pig or rabbit skins have no adequate barrier function comparable to that of human skin. The use of cultured or reconstructed skin models is under development and those systems are not yet advised for *in vitro* testing on the basis of their insufficient barrier function [Coquette et al. 2000].

The origin of skin samples should be specified in the respective report. The following information is required:

- Species: by preference human or pig skin should be used. Although rodent skin is not representative for human skin [ECETOC 1993], the results of such studies will not be disregarded, since these models produce an overestimation of the dermal absorption (worst case).
- Skin location: abdomen, leg or breast (human skin); abdomen, breast, back, flanks or ears (pig skin).
- Gender and age: although these factors are not believed to be important variables, they should be stated.
- Fresh/frozen skin: when significant biotransformation of the test compound in the skin is expected, freshly excised, viable skin should be used [Diembeck et al. 1999].
- Details on preservation and storage conditions of the skin should be specified (e.g. skin can be stored in aluminium foil at -20°C or lower) [Howes et al. 1996, Bronaugh et al. 1986]. During transport skin samples should be kept at or below 4°C.

Skin samples that may be used are split-thickness (200-500 µm) or full-thickness (500-1000 µm) skin preparations [Sanco/222/2000]. Dermatomed skin is often used. Skin thickness should be measured by an appropriate method, which should be described in the report. The skin samples should be prepared to fit the experimental cell.

- For human skin: split-thickness skin should be the general rule. If for a specific reason, full-thickness is required, this should be justified.
- For pig skin: since it is technically more difficult to obtain intact split-thickness skin, this could justify the use of full-thickness skin.

When epidermal membranes are used for the *in vitro* dermal absorption study, the reason for this should be justified. Epidermal membranes are sometimes quite fragile and some mass balance techniques (e.g. tape stripping) cannot be applied to this model. It must also be mentioned that epidermal membranes may overestimate human *in vivo* skin absorption [Van de Sandt et al. 2000].

The minimum skin area to be covered is considered to be 0.64 cm<sup>2</sup>.

#### 4.4. Skin integrity

Barrier integrity is crucial for the experiment, and must therefore be measured. This is achieved by either measuring the penetration of a marker molecule, e.g. tritiated water, caffeine or sucrose, or by physical methods, such as determination of TEWL (Transepidermal Water Loss) or TER (Transcutaneous Electrical Resistance). Data obtained should be reported.

#### 4.5. Skin temperature

Because the rate and extent of skin absorption is temperature-dependent, the skin disc temperature should be maintained constant ( $32 \pm 1^\circ\text{C}$ , corresponding to the normal human skin surface temperature). The method of temperature maintenance should be described in the report.

#### 4.6. Test substance

The relevant physical and chemical data (e.g. MW,  $\log P_{ow}$ , solubility, stability, and  $pK_a$  of the test substance) should be given.

The purity of the test substance should be described and should be comparable to that of the substance in marketed products (see 4.10). It is recognised that radio-synthesis of [ $^{14}\text{C}$ ]- or [ $^3\text{H}$ ]-labelled substances may result in a somewhat different purity and/or impurity profile than that of substances produced by large-scale chemical production.

As mentioned under 4.2, the solubility and stability of the test substance in the receptor fluid for the entire test duration should be documented.

A separate set of criteria dealing with dermal absorption issues related to nanoparticles/nanotubes will, if necessary, become available as an annex to this Opinion.

#### 4.7. Preparation of the dose and vehicle / formulation

The dose and vehicle / formulation should be representative for the in use condition(s) of the finished cosmetic product. The quantitative composition of every formulation used during the experiment, should be given.

More than one concentration of the test substance, including the highest requested one, is used in typical formulations spanning the range of human exposure. These concentrations should be selected in such a way that the range of the linear curve of concentration versus dermal absorption is demonstrated.

The stability of the test substance under the foreseeable conditions of application and usage must be ascertained.



#### 4.8. Dose and volume of test substance

The dose of the test formulation as well as its contact time (exposure) with the skin should resemble use conditions. The amount of the formulation to be applied is adapted to the consumer use values described in the Notes of Guidance [SCCNFP/0690/03], and usually is between 2-5 mg/cm<sup>2</sup> for solids and semi-solid preparations, and up to 10 µl/cm<sup>2</sup> for liquids. For oxidative hair dye formulations, 20 mg/cm<sup>2</sup> is applied. Deviations should be explained. The volume of formulation used should be appropriate to spread the sample homogeneously over the skin surface. This depends on the viscosity and lipophilicity of the formulation. Both mass and volume applied should be stated in the test report.

#### 4.9. Study period and sampling

The exposure time and sampling period(s) should be defined in the protocol. The normal exposure time is 24 hours with regular sampling intervals. Longer duration of the study may result in membrane deterioration and requires careful control of membrane integrity. The exposure time should be consistent with the intended use of the cosmetic formulation. E.g. for oxidative hair dye formulations, the time of contact could vary from 15 to 45 minutes or even more according to the in-market use. The skin surface will also be rinsed using a procedure mimicking the consumer situation. Sampling of the receptor fluid is continued until e.g. 24 hours.

The frequency of sampling should be chosen adequately to allow the determination of the extent/rate of absorption and the absorption profile. Kinetic measurements have to be obtained for at least 6 post-application time points, including one early time point (30 minutes), in order to be able to estimate the absorption kinetics. For rinse-off products, measurements after rinsing have to be taken. The full sampling procedure must be described in the report.

#### 4.10. Analytical methods

Appropriate analytical techniques, e.g. scintillation counting, HPLC or GC, should be used. Their validity, sensitivity and detection limits should be documented in the report. When an increase of sensitivity is needed, the test substance should, whenever possible, be radio-labelled.

Qualitative or semi-quantitative methods, such as micro-autoradiography, may be useful tools for skin distribution assessments.

#### 4.11. Data collection

The test compound must be determined in the following compartments:

- Product excess on the skin (dislodgeable dose)
- Stratum corneum (e.g. adhesive tape strips)
- Living epidermis (without stratum corneum)
- Dermis
- Receptor fluid

For the reason of an appropriate mass balance, it is necessary to check for substance adsorbed to the equipment (included in rinsing solutions and/or compartments).

#### 4.12. Mass balance analysis / recovery

The mass balance of the applied dose must be determined.

The overall recovery of test substance (including metabolites) should be within the range of 85-115%. Lower or higher recovery rates should be investigated and/or explained.

#### 4.13. Variability / validity / reproducibility

- The variability of dermal absorption studies depends on the penetration rate of a particular ingredient; the lower the penetration rate, the higher the variability. This high variability is due to known intra-individual and inter-individual characteristics of the stratum corneum barrier. The relative variability of the method should be documented.  
Experience has shown that the high variation in dermal absorption may partly be explained by differences in skin samples. Therefore skin samples should be taken from a suitable anatomical site and the study should use a sufficient number of samples and replicates.
- The technical ability of the performing laboratory and the validity of the method used should be assessed at regular intervals, at least twice per year, by using reference compounds like caffeine or benzoic acid. These data should be included in the study report [OECD 2004, Van de Sandt et al. 2004].
- The results of the dermal absorption studies should be reproducible. A minimum of **six** evaluable samples (human or pig skin), from each of at least **three** donors, should be used per dose tested. The coefficient of variation should be less than 30 %. If statistical evaluation is not possible, the highest observed penetration value will be used in the systemic exposure dosage (SED) calculation.
- For oxidative hair dyes, the relevant combination(s) of hair dye, coupler(s) and developer(s) should be tested.

## 5. RESULTS

Dermal absorption should be expressed as an absolute amount [ $\mu\text{g}/\text{cm}^2$  of skin surface] and as a percentage of the amount of test substance contained in the intended dose applied per square centimetre of skin surface.

The amounts of penetrated substance(s) found in the receptor fluid are considered to be systemically available. The epidermis (except for the stratum corneum) and dermis are considered as a sink, therefore the amounts found in these tissues are considered as absorbed and are added to those found in the receptor fluid. The amounts that are retained by the stratum corneum at the time of sampling are not considered to be dermally absorbed, and thus they are not expected to contribute to the systemic dose.

The absorption rate and mass balance should be calculated separately for each diffusion cell. Only then, the mean  $\pm$  S.D. and median with 10% and 90% percentiles should be calculated.

All measurements, statistical processing and obtained kinetic curves have to be provided.

In case the results are derived from an inadequate *in vitro* study, the default value of 100% absorption is applied.

## 6. REFERENCES

**2000/6/EC** - Twenty-fourth Commission Directive 2000/6/EC of 29 February 2000 adapting to technical progress Annexes II, III, VI and VII to Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. *Official Journal L 56, 01/03/2000 p.42.*

**Beck H.**, Benech-Kieffer F., Bracher M., Diembeck W., Dupuis J., Lovell W., Steiling W. Cosmetic ingredients : Guidelines for percutaneous absorption / penetration. COLIPA Guidelines, 1-36 (1995)

**Bronaugh R.L.**, Stewart R., Simon M. Methods for *in vitro* percutaneous absorption studies VII : use of excised human skin. *Journal of Pharmaceutical Sciences* 75, 1094-1097 (1986).

**Coquette A.**, Berna N., Poumay Y., Pittelkow M.R. The keratinocyte in cutaneous irritation and sensitisation. In : *Biomedical Modulations of Skin Reactions in Dermal and Transdermal Drug Delivery*, Chapter 8. CRC Press, pp.125-143 (2000)

**Diembeck W.**, Beck H., Benech-Kieffer F., Courtellemont P., Dupuis J., Lovell W., Paye M., Spengler J. and Steiling W. Test guidelines for *in vitro* assessment of dermal absorption and percutaneous penetration of cosmetic ingredients. *Food & Chemical Tox.*, 37, 191-205 (1999).

**Directive 98/8/EC** of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market *Official Journal L 123, 24/4/98 p. 1.*

**ECB** (European Chemicals Bureau) Technical Guidance Document on Risk Assessment in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Doc. EUR 20418 EN/1, European Communities (2003).

**ECETOC** Percutaneous absorption. European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), Monograph No 20, Brussels (1993).

**Howes D.**, Guy R., Hadgraft J., Heylings J., Hoeck U., Kemper F., Maibach H., Marty J-P., Merk H., Parra J., Rekkas D., Rondelli I., Schaefer H., Täuber U. and Verbiere N. Methods for assessing percutaneous absorption, ECVAM Workshop Report n. 13. *Alternatives To Laboratory Animals* 24, 81-106 (1996).

**OECD 427** - OECD Guideline for testing of chemicals - Guideline 427 : Skin absorption: *In vivo* method Organization for Economic Cooperation and Development, Paris, adopted 13 April 2004.

**OECD 428** - OECD Guideline for testing of chemicals - Guideline 428 : Skin absorption: *In vitro* method Organization for Economic Cooperation and Development, Paris, adopted 13 April 2004.

**OECD** Guidance Document for the Conduct of Skin Absorption Studies. Organization for Economic Cooperation and Development (OECD), Environment Directorate, OECD Environmental Health and Safety Publications, Series on Testing and Assessment No. 28, Paris (2004).

**Pauwels M.** and Rogiers V. How to improve the quality of a European cosmetic ingredients' dossier? Oral presentation given during the Scientific Forum of the COLIPA (The European Cosmetic Toiletry and Perfume Association) General Assembly 2004, Amsterdam, 24/06/2004.

**Ross J.H.**, Dong M.H. and Krieger M.I. Conservatism in pesticide exposure assessment. *Regulatory Toxicology and Pharmacology* 31, 53-58 (2000).

**Sanco/222/2000** – Guidance Document on Dermal Absorption European Commission, Health and Consumer Protection Directorate-General Doc. Sanco/222/2000 rev 6, of 27 November 2002.

**SCCNFP/0167/99, Final** : Basic Criteria for the *in vitro* assessment of percutaneous absorption of cosmetic ingredients, *adopted by the SCCNFP during the 8<sup>th</sup> plenary meeting of 23 June 1999.*

**SCCNFP/0690/03, Final** : Notes of Guidance for Testing of Cosmetic Ingredients for Their Safety Evaluation, *adopted by the SCCNFP during the 25<sup>th</sup> plenary meeting of 20 October 2003.*

**SCCNFP/0750/03, Final** : Basic Criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients, updated October 2003, *adopted by the SCCNFP during the 25<sup>th</sup> plenary meeting of 20 October 2003.*

**Schaefer H.** and Redelmeier T.E. *Skin Barrier, Principles of Percutaneous Absorption*, Karger, Basel (1996).

**US EPA** (United States Environmental Protection Agency) Proposed rule for *in vitro* dermal absorption rate testing of certain chemicals of interest to occupational safety and health administration. *Federal Register*, Volume 64, Number 110, June 9 (1999).

**Van de Sandt J.J.M.**, Meuling W.J.A., Elliott G.R., Cnubben N.H.P. and Hakkert B.C. Comparative *in vitro* – *in vivo* percutaneous absorption of the pesticide propoxur. *Toxicological Sciences* 58, 15-22 (2000).

**Van de Sandt J.J.M.**, vanBurgsteden J.A., Carmichael P.L., Dick I., Kenyon S., Korinth G., Larese F., Limasset J.C., Maas W.J.M., Montomoli L., Nielsen J.B., Payan J.-P., Robinson E., Sartorelli P., Schaller K.H., Wilkinson S.C., Williams F.M. In vitro predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study. *Regul Toxicol Pharmacol* 39, 271-281 (2004).

**WHO** (World Health Organisation) Kielhorn J., Melching-Kollmuß S., Mangelsdorf I. Dermal Absorption. WHO / IPCS Environmental Health Criteria, Draft February 2005, accessible through [http://www.who.int/ipcs/methods/dermal\\_absorption/en/](http://www.who.int/ipcs/methods/dermal_absorption/en/) (consulted Dec 2005).

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## Guidelines for Percutaneous Absorption/Penetration

Edition of 1997



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# 1. BACKGROUND

The 6th Amendment to the Cosmetics Directive (93/35/EEC) includes a potential ban on the use of animals in the testing of cosmetic products and their ingredients, from January 1, 1998.

With regard to the assessment of cutaneous absorption/penetration properties of substances, in vitro methodologies are recommended for ethical reasons and feasibility. Excised and reasonably standardised skin is easily obtainable from various species whereas human skin from individuals is available only under favourable circumstances and in limited quantities from surgical or post-mortem sources.

The value of organotypic skin models for penetration studies has to be demonstrated by further research. The viability of the skin is not a pre-requisite for penetration testing, since the process depends on passive diffusion and not on active transport. Furthermore, biotransformation is of minor interest and importance in the majority of cases. In the exceptional case of relevance, biotransformation can be monitored.

## 2. INTRODUCTION

Percutaneous penetration is the passage of material from the stratum corneum surface of the skin to the systemic circulation. Percutaneous absorption is the passage of topically applied materials into the skin.

In vitro methods used now in many laboratories, utilise transport across full- or split-thickness animal or human skin to a receptor fluid reservoir. It is possible to estimate in vivo absorption by extrapolating from suitable in vitro data.

This guideline for the testing of percutaneous penetration in vitro describes a general procedure for measuring the penetration of test substances through excised mammalian skin. The barrier properties of skin are usually maintained after excision from the body and appropriate storage in a freezer for up to 3 months, since penetration is driven by passive diffusion and there is no evidence for active transport (ref. 5). Therefore, skin viability is not a prerequisite for these studies. The principal diffusion barrier has been identified as the stratum corneum, the integrity of which has to be controlled.

Details of the procedures are presented in the form of examples, in the appendix (section 9). The appendix contains examples of the following standard protocols, according to a ranking in terms of relevance to mimic human conditions (ref. 6):

- human skin
- pig skin
- rat skin



### 3. INITIAL CONSIDERATIONS

The in vitro methods described in these guidelines provide information in all cases where the stratum corneum is the rate limiting barrier to percutaneous absorption and are, therefore, applicable to those compounds which are sufficiently soluble in the intended receptor fluid. There is considerable experience to allow these in vitro methods to be used for the assessment of percutaneous absorption/penetration and for the prediction of the in vivo situation (ref. 1). The use of appropriately prepared skin membranes (full thickness, split-thickness and isolated epidermis) may allow the measurement of the amount of substance delivered to the systemic circulation and the amount delivered to the epidermis or dermis (ref. 6).

The advantages of the in vitro method are that it can be used with skin from human and other species, it does not need live animals and it is suitable for compounds extensively metabolised in the body. The limited supply of human skin means that the routine use of this material is unlikely and hence animal skin will normally be used. There are now many comparisons in the literature for predictions of in vivo absorption/penetration from in vitro data to be made with some confidence for the majority of compounds (ref. 1 to 4).

### 4. PRINCIPLE OF THE TEST

The test substance, either as such or dissolved in an appropriate solvent or galenic formulation, thereby yielding the test sample, is applied to the intact surface of the skin disk separating the upper and lower chambers of a diffusion cell, which can be run in static or flow-through mode. The test sample remains in contact with the skin on the donor side for a defined period of time (leave-on or rinse-off respectively, depending on the intended use conditions). The receptor fluid is sampled once at the end of the experiment or at various time points in between. The skin and/or fluid samples are analysed by an appropriate method (e.g. scintillation counting, HPLC, GC). The integrity of the barrier should be checked by an appropriate method (see paragraph 5.4).

### 5. DESCRIPTION OF THE METHOD

#### 5.1 Penetration cell design

The penetration cell consists of the upper donor and the lower receptor chamber, separated by a skin membrane. The epidermis faces the donor chamber and the lower surface the receptor chamber. The cells must be made from an inert and non-absorbing material (e.g. glass or PTFE). Temperature control is crucial throughout the experiment and it should be maintained at in vivo skin conditions (see paragraph 6.3). The receptor fluid must be well-mixed throughout the experiment. Sampling should be feasible without interrupting the experiment, by appropriate cell design.





## 5.2 Receptor fluid

The composition of the receptor fluid should not limit the extent of penetration of the test substance, i.e. the theoretical total solubility should be guaranteed. The receptor fluid must not affect the integrity or alter the permeability properties of the skin. A saline or buffered saline solution is recommended for hydrophilic compounds. For lipophilic molecules, addition of serum albumin or other appropriate solutors, such as non-ionic surfactants, is recommended. The appropriate receptor fluid volume is determined by the solubility and analytical detectability of the test substance. It can be adapted by choosing a receptor chamber of adequate volume (static) or by varying the setting of the pump (flow-through).

## 5.3 Skin disks

The test should be carried out with an appropriate number (i.e. minimum six) of skin disks of similar integrity. Split-thickness skin (epidermis and upper dermis obtained by dermatomisation or epidermis only obtained by pre-treatment with heat, enzymes or chemicals) may be used. Full thickness skin may be used provided that the skin compartments are analysed in detail. Details on preparation are found in the standard protocols. In case of lipophilic compounds split-thickness skin ( $\leq 1$ mm thick layer) or epidermal preparations are to be preferred to limit the dermal retention in vitro. The skin should be clipped before preparing membranes. Skin thickness should be measured by an appropriate method.

## 5.4 Membrane integrity

Since barrier integrity is crucial for the experiment, it should be checked by measuring the skin penetration of a marker molecule, e.g. tritiated water, for which suitable historical control data are available. Alternatively, the integrity can also be tested with a non-radioactive probe, e.g. caffeine, or with physical methods like TEWL or TER (Transepidermal Water Loss or Transdermal Electrical Resistance respectively). Details of such procedures are given in the appendix.

## 5.5 Test substance

Toxicological and physico-chemical data, purity, solubility, stability, octanol-water partition coefficient and analytical methods and their detection limits should be known for the test substance. Relevant information on the formulation in which the test substance is used should be available.

## 5.6 Preparation of the dose

Depending upon the purpose of the experiment, the test substance may be used as such, in solution or in formulation, where appropriate, to allow good contact with the skin or to simulate the intended use conditions. The stability of the test sample and substance under the proposed conditions of administration and usage should be known.



# 6. PROCEDURE

## 6.1 Application of test substance

The dose as well as the contact time with the skin should mimic the intended use conditions as closely as possible or be higher to obtain relevant safety data under exaggerated use conditions (for assessment of foreseeable misuse). The amount of the formulation to be applied should be adapted whenever possible to the consumer use values published by Colipa (doc. 93/067). If permeability constants are being determined then infinite dose conditions are required.

## 6.2 Fluid dynamics

The receptor fluid, preferably degassed (e.g. by sonication), must be thoroughly stirred at all times or be continuously replaced in flow through chambers. The choice of static or flow-through conditions in the receptor cell should be made on a compound-by-compound basis, depending on its absorption properties and the goal of the study. It must be ensured that the amount of penetrant in the receptor fluid is less than 10% of its saturation level, in order to prevent significant back penetration and hence underestimation of absorption.

## 6.3 Temperature

Because the rate and extent of skin absorption is temperature dependent, the skin temperature should be maintained constant ( $30-32^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) either by use of penetration chambers with water jackets or temperature-controlled incubators.

## 6.4 Study time

The study time is determined by the subsequent reasonable characterisation of the absorption profiles and the intended use conditions. The exposure time and sampling period should be defined in the protocol. A normal study time of 24 hours is recommended.

## 6.5 Sampling

The frequency of sampling will depend on the rate and extent of dermal penetration, but should be sufficient to allow the extent or rate of penetration and/or the profile to be determined.

Where appropriate, the surplus of non-penetrated test substance should be determined in skin samples, rinsings and cell washings. The overall recovery of test substance should be at least 85%. If low recoveries of the test substance are obtained with all cells, a search should be conducted to determine the cause(s) which may include binding to proteins, to penetration cell surface and tubings, as well as possible evaporation or loss by chemical reaction.

## 6.6 Analysis

The receptor fluid must be analysed and, if possible, an analysis should be made of the amounts found in individual skin layers and on the skin surface. Suitable analytical procedures must be used, e.g. scintillation counting, HPLC or GC.

# 7. DATA REPORTING

## 7.1 Data

The absorption profile normally is determined up to 24 hours post application with usually six cells of similar barrier integrity. When adequate data are available, the lag time and the absorption rates may be calculated. Permeability constants can only be calculated from infinite dose experiments.

## 7.2 Test report

The test report should include the following information:

- Test substance:
  - chemical structure, physico-chemical properties and purity;
  - identification data.
- Vehicle (if appropriate):
  - type of formulation;
  - justification for choice of formulation.
- Test conditions:
  - source and site of skin sample, method of preparation, integrity;
  - details of test sample preparation, final concentration, stability and homogeneity of the preparation, especially when blended with radiolabelled test substance;
  - details of the administration of the test substance (exposure time and conditions, e.g. occlusion or not, rinse-off, leave-on);
  - justification for choice of skin source and skin preparation;
  - analytical method, sample clean-up, recovery and detection;
  - overall recovery of material, if possible.
- Reporting of results:
  - tabulation of individual results at each time point;
  - lag times, absorption rates, absorption profiles, if appropriate.

Data can be presented in various ways. Depending on the experimental set-up, percent dose, micrograms, micromoles or a penetration constant can be displayed. The presentation of cumulated percent dose or micrograms/cm<sup>2</sup> in tables and graphs is recommended. Graphs are prepared with appropriate computer programs which may be equipped with functions for statistical treatment of data.

- Statistical methods
- Discussion of results and conclusions:  
It should be remembered that percentage absorbed values are dependent on specific exposure and formulation conditions. In some cases, a permeability constant may provide useful interpretation of the results.

### 7.3 Implications for safety evaluation

Percutaneous absorption/penetration is a key parameter for the safety evaluation of cosmetic ingredients, as well as of the finished products containing them. Determination of absorption/penetration allows the interpretation of systemic toxicity data in terms of exposure via topical application. Furthermore, the utilisation of the procedures described in this guideline will provide a suitable substitute for in vivo experiments to measure percutaneous absorption/penetration. Depending on the outcome of such studies, with the context of the overall safety evaluation, further animal toxicity studies would be minimised. The results of such studies may also provide guidance on the optimisation of the design of further required tests.

## 8. REFERENCES

1. Scott R.C. & Ramsey J.D., *J. Investig. Dermatol.*, 89, pp. 142-146, 1987.
2. Scott R.C., in *Biological Monitoring for Pesticide Exposure: Measurement, Estimation*, 1989.
3. Grissom R.E., in *Prediction of Percutaneous Penetration - Methods, Measurements, Modelling*, (Eds. Scott R.C., Guy R.H. and Hadgraft J.), *IBC Technical Services*, London, pp. 73-83, 1990.
4. Bronaugh R. L., Stewart R. & Simon M., *Methods for in vitro percutaneous absorption studies VII: use of excised human skin*, *J. Pharm. Sci.*, 75, 11, 1986.
5. Bartnik F.G., Reddy A.K., Klecak G., Zimmermann V., Hostynek J.J., Künstler K., *Percutaneous absorption, metabolism and hemolytic activity of n-Butoxyethanol*, in *Fundam. and Applied Tox.*, 8, 59-70, 1987.
6. *ECETOC Monograph N° 20*.

# STANDARD PROTOCOL

## PERCUTANEOUS ABSORPTION/ PENETRATION *IN VITRO* EXCISED HUMAN SKIN

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# I. INTRODUCTION

This standard protocol describes in vitro percutaneous absorption/penetration studies of cosmetic ingredients through human skin. Such studies are performed to measure the amount of test compound penetrating through the skin barrier for safety evaluation. Recommendations published by an AAPS-FDA panel on percutaneous absorption studies of drugs have been taken into account for this protocol (ref. 1).

The in vitro percutaneous absorption/penetration of the test compound is assessed using samples of isolated human skin placed in static or dynamic diffusion cells (ref. 2,3,4).

All the mentioned SOP (Standard Operating Procedure) have been specifically written up for the respective laboratory.

## II. MATERIALS

- Dynamic mode: flow-through diffusion cell (ref. 3)
- Static mode: static Franz diffusion cell (ref. 2)

Usual laboratory equipment as indicated in a specific SOP.

## III. METHODS

### III.1. Preparation of the formulation

#### III.1.1. UNLABELLED TEST COMPOUNDS

The specifications of each test compound must be defined prior to the formulation: a recent analysis of the respective batch by the analytical department must be included in the final study report.

The qualitative controls of the formulation are performed by the formulator.

The quantitative controls are performed according to the SOP “*Control of an unlabelled formulation*”. This control is based on HPLC or another suitable method in order to ascertain the concentration of the test compound in the formulation.

#### III.1.2. RADIOLABELLED TEST COMPOUNDS

The degree of radiolabelling is specific for each formulation: the radiolabelled test compound is diluted according to its initial specific activity.



The supplier of the radiolabelled compound must provide a complete analysis bulletin including the radiochemical purity and date of the analysis; radiochemical purity must be reassessed before beginning the formulation and in the final formulation by the technical investigator; analytical bulletins should be included in the final study report.

Since only small quantities of radiolabelled formulations can be prepared each time, unlabelled pilot formulation assays on the respective scale should be performed and analytical results should be provided by the study promotor/department requesting the study. The latter ascertains the feasibility of the formulation and its stability. The formulation should remain stable for the duration of the study.

The qualitative controls of the formulation are performed by the formulator.

The quantitative controls are performed according to a specific SOP. This control is based on HPLC or another method suited to ascertaining the concentration of the test compound (radiolabelled and unlabelled) in the formulation.

Specific radioactivity of the formulation is determined by scintillation counting using an external standard; the quench curves are established according to a specific SOP.

## **III.2. Preparation of the samples of human skin**

### III.2.1. ORIGIN AND STORAGE OF BIOLOGICAL SAMPLES

Human skin used for these experiments is obtained from surgery. The skin samples can be obtained from a variety of anatomical sites including the breast (mastectomy and reduction mammoplasty) and abdomen (cosmetic reductive surgery). Skin must be collected as soon as possible after surgery. All skin samples are checked visually to ensure they are healthy and unaltered by clinical removal conditions. For transportation from the hospital/clinic to the laboratory, the skin should be kept in an isotherm container at 4°C. In the laboratory, each skin sample should be identified (identification number, type, age, gender, date of operation) and have the subcutaneous fat removed. The whole process of transporting and preparing the skin samples must be achieved as quickly as possible.

The skin samples are put into plastic bags, sealed and stored at - 20°C until use. Skin samples can be kept in a freezer for up to 3 months. For in vitro percutaneous absorption/penetration experiments, it has been shown that human skin permeability is unaffected by freezing (ref. 5, 6). These different stages should be performed according to a specific SOP *“including security measures for handling of biological materials”*.

### III.2.2. PREPARATION OF SKIN DISCS

An in vitro study using isolated skin should simulate as closely as possible the in vivo conditions. In vivo, a compound must diffuse up to the upper papillary dermis before being taken up by blood vessels and then entering the systemic circulation. Thus, depending on the solubility of the test

compound, to limit the effect of the dermal retention in vitro, especially with hydrophobic compounds, split-thickness skin (dermatomed skin or isolated epidermis) should be used (ref. 7).

Each skin sample is thawed at room temperature and prepared the day of the experiment according to a specific SOP:

- ***Dermatomed skin:***

An electric dermatome (Davies simplex, THACKRAY SURGERY) is used to cut horizontal slices of skin. The thickness of the cut is controlled by a lever on the side of the dermatome head with the indicated calibrations. Full-thickness skin is fixed on a dissection board, epidermal side up, and sections are cut at 300 to 500  $\mu\text{m}$ . The thickness of the membrane obtained is the result of the pressure applied and the angle of the dermatome as it is pushed across the skin. During this procedure, a constant angle of inclination of the oscillating blade relative to the skin must be maintained. The dermatomed layer thus obtained includes the epidermis and some dermal tissue.

- ***Isolated epidermis:***

Epidermal membranes are prepared from full-thickness skin by a heat separation method (ref. 8). Water is heated in a beaker to 58-60°C. Full-thickness skin is suspended in the water with forceps for 60 seconds. Then the epidermis is gently peeled off from the dermis using forceps. The remaining epidermal membrane is cut to fit the diffusion cell area. Each epidermal membrane should be checked for integrity with the aid of a stereomicroscope.

### III.2.3. CONTROL OF SKIN DISC THICKNESS

Skin thickness should be measured with a Digitaler Meßtaster (MT12, HEIDENHAIN) and a bidirectional counter (VRZ 401, HEIDENHAIN) or with equivalent equipment. The measurements should be performed according to the directions for use. Ten measurements per skin disc should be made. This process should be performed according to a specific SOP.

### III.2.4. SPECIFIC RECOMMENDATIONS FOR THE USE OF HUMAN SKIN

As there is wide variability in human skin permeability (ref. 9), not less than 3 skin donors should be used to take account of interindividual variability besides the intraindividual variability; a proper statistical design should be used.

Age and anatomic site influence percutaneous absorption (ref. 9,10). Thus, skin samples from the same anatomical region and in the same age range (20-60 years) should be used to limit these variations.

To preserve the integrity of human skin membranes mounted in diffusion cells, the study time should never exceed 24 hours.



### **III.3. Preparation of the diffusion cells**

The number of diffusion cells per experiment and the number of runs per study, must be specified in the protocol. The study should involve a minimum of six samples of similar integrity. This process should be performed according to a specific SOP.

#### **III.3.1. STATIC MODE OR DYNAMIC MODE**

The choice of static mode or dynamic mode depends on the absorption/penetration properties of the test compound and depends on the aim of the study.

The skin samples are placed as a barrier between the two halves of the diffusion cell, the stratum corneum facing the donor chamber; the donor side of the cell is open to the air (non occlusive conditions). The skin sample is kept at (32 +/- 1)°C by circulation of temperature controlled receptor fluid through the cell (dynamic diffusion cell) or by immersing the cell (static diffusion cell) in a temperature-controlled water bath.

The receptor chamber is filled with receptor fluid, capped and allowed to attain the correct temperature 1 hour before the beginning of the experiment. In dynamic mode, care is taken to ensure that no air bubbles form on the underside of the skin throughout the experiment.

#### **III.3.2. RECEPTOR FLUID**

To maintain the integrity of the skin samples and to collect the test compound, the receptor fluid is saline or buffered saline solution; a non-ionic surfactant can be added to solubilise the test compound especially for lipophilic compounds (ref. 11).

Before the experiments, it should be checked that the test compound is stable in the receptor fluid (in experimental conditions) for a period corresponding to the duration of the experiment.

### **III.4. Test of skin integrity**

Each sample of human skin must be checked for integrity before the application of the test compound. This test must not affect the quality of the skin samples and must not influence the penetration of the test compound.

The integrity of the skin samples is checked with the the aid of a stereomicroscope and/or by measuring TEWL (Trans Epidermal Water Loss) (ref. 12). The procedure is performed according to a specific SOP.

### III.5. Application of formulations

The quantities to be applied are expressed as follows:

- amount of formulation applied ( $\text{mg}/\text{cm}^2$ );
- amount of test compound applied ( $\mu\text{g}/\text{cm}^2$ );
- in case of radiolabelled compounds: amount of radioactivity applied ( $\text{Bq}/\text{cm}^2$ ).

A finite dose of the test compound is applied to the skin surface (ref. 13) i.e. about  $2 \text{ mg}/\text{cm}^2$  of a semisolid formulation (cream, ointment, gel, etc.) and about  $5 \mu\text{l}/\text{cm}^2$  of liquid preparations (solutions, emulsions, etc.). If semisolid formulations are compared to liquid formulations, identical amounts of  $5 \text{ mg}/\text{cm}^2$ . When investigating rinse-off preparations (e.g. hair dyes), an infinite dose ( $> 10 \text{ mg}/\text{cm}^2$ ) of the test compound is applied to the skin surface to mimic the use conditions. The rinsing procedure must be specified in the protocol.

The application is performed according to a specific SOP.

### III.6. Start of the experimental setup and collection of fractions

The experiments are started immediately after the application of the formulation, according to a specific SOP.

In the static mode, aliquots of the receptor fluid are taken either throughout the experiment or at the end of the experiment. In the dynamic mode, the receptor fluid is pumped from a reservoir into and through the cell by a peristaltic pump. After exiting the cell, the receptor fluid is collected in an automatic fraction collector.

### III.7. Study time

The study time is chosen according to the nature and intended use of the test compound. Rinse-off preparations are left on the skin for a restricted time (e.g. 30 minutes in the case of hair dyes). Then the skin and the upper part of the diffusion cell are rinsed and the experiment is continued for a maximum of 24 hours. Leave-on preparations are left in place for the entire study time (maximum 24 hours).

The diffusion cells are then taken apart according to a specific SOP.

### III.8. Analysis

In all cases, the receptor fluid samples are analysed. In case of dermatomed skin, the epidermis is separated from the dermis and the remaining dermis sample is analysed.

### III.8.1. UNLABELLED TEST COMPOUNDS

If relevant and if a suitable analytical method exists (in terms of detection limit, sensitivity, etc) an analysis will be made of the amounts of the test compound on the skin surface, in rinsings, cell washings and skin layers. The preparation of samples, extraction steps and analysis by HPLC or another suitable method depend on the test compound and should be described in the appendix of the study report.

This procedure should be performed according to a specific SOP.

### III.8.2. RADIOLABELLED TEST COMPOUNDS

An analysis of the test compound on the skin surface, in rinsings, cell washings and skin layers should be made. This procedure should be performed according to a specific SOP. The analyses are carried out by means of scintillation counting according to a specific SOP. Scintillation counting is performed using an external standard; the quench curves are established according to a specific SOP.

## III.9. Presentation of the results

The results are expressed in  $\mu\text{g}/\text{cm}^2$  and % of the applied dose in the form of tables and graphs.

## III.10. Interpretation of the results for safety evaluations

Classical in vitro percutaneous absorption studies only consider as penetrated the amount of compound entering into the receptor fluid. This interpretation is only correct when the skin samples are completely devoid of dermal tissue, i.e. when the epidermis has been removed from the dermis by heat (in vivo the dermis is vascularised, i.e. is part of the central compartment).

The dermatome-based separation technique leaves some upper dermal tissue underneath the epidermis. Hence, the epidermal and the dermal compartments have to be considered separately. The amount of test compound found after the study time in the receptor fluid, plus that found in the dermis, must be considered to have crossed the skin barrier i.e. as penetrated.

## III.11. Study report

The study report has to be finalized according to a specific SOP.

## III.12. Archives

The protocol, raw data and study report are kept on file by the study director's office for at least 5 years.

## LITERATURE REFERENCES:

1. Skelly J.P., Shah V.P., Maibach H.I., Guy R.H., Wester R.C., Flynn G. & Yacobi A., *FDA and AAPS report of the workshop on principles and practices of in vitro percutaneous penetration studies: Relevance to bioavailability and bioequivalence*, Pharm. Res., 4, p. 3, 1987.
2. Franz T.J., *Percutaneous absorption: On the relevance of in vitro data*, J. Invest. Dermatol., 64, pp. 190-195, 1975.
3. Gummer C.L., Hinz R.S. & Maibach H.I., *The skin penetration cell: a design update*, Int. J. Pharm., 40, pp. 101-104, 1987.
4. Addicks W.J., Flynn G.L. & Weiner N., *Validation of a flow-through diffusion cell for use in transdermal research*, Pharm. Res., 4, 1987.
5. Harrison S.M., Barry B.W. & Dugard P.H., *Effects of freezing on human skin permeability*, J. Pharm. Pharmacol., 36, pp. 261-262, 1984.
6. Bronaugh R.L., Stewart R.F. & Simon M., *Methods for in vitro percutaneous absorption studies VII: Use of excised human skin*, J. Pharm. Sci., 75, p. 11, 1986.
7. Collier S.W. & Bronaugh R.L., *Preparation of human and animal skin, In vitro percutaneous absorption: Principles, Fundamentals and applications*, (Bronaugh R.L. & Maibach H.I., Eds.), CRC Press, Boca Raton, 1991.
8. Kligman A.M. & Christophers E., *Preparation of isolated sheets of human stratum corneum*, Arch. Dermatol., 88, pp. 702-705, 1963.
9. Wester R.C. & Maibach H.I., *Individual and regional variation with in vitro percutaneous absorption, In vitro percutaneous absorption: Principles, Fundamentals and Applications*, (Bronaugh R.L. and Maibach H.I., Eds.), CRC Press, Boca Raton, 1991.
10. Marzulli F.N. & Maibach H.I., *Permeability and reactivity of skin related to age*, J. Soc. Cosmet. Chem., 35, pp. 95-102, 1984.
11. Collier S.W. & Bronaugh R.L., *Receptor fluids, In vitro percutaneous absorption: Principles, Fundamentals and Applications*, (Bronaugh R.L. and Maibach H.I., Eds.), CRC Press, Boca Raton, 1991.
12. Lotte C., Rougier A., Wilson D.R. & Maibach H.I., *In vivo relationship between transepidermal water loss and percutaneous penetration of some organic compounds in man: effect of anatomic site*, Arch. Dermatol. Res., 279, 1987.
13. Franz T.J., *The finite dose technique as a valid in vitro model for the study of percutaneous absorption in man*, Currents problems in Dermatology, 7, (Simon G.A., Paster, Klingberg M.A. & Kaye M., Eds.), Karger, Basel, 1978.