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Suitability of skin integrity tests for dermal absorption studies in vitro

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ABSTRACT

Skin absorption testing in vitro is a regulatory accepted alternative method (OECD Guideline 428). Different tests can be applied to evaluate the integrity of the skin samples. Here, we compared the pre- or post-run integrity tests (transepidermal electrical resistance, TEER; transepidermal water loss, TEWL; absorption of the reference compounds water, TWF, or methylene blue, BLUE) and additionally focused on co-absorption of a ^{3}H-labeled internal reference standard (ISTD) as integrity parameter. The results were correlated to absorption profiles of various test compounds. Limit values of 2 kΩ, 10 g m^{-2} h^{-1} and 4.5 $*$ 10^{-3} cm h^{-1} for the standard methods TEER, TEWL and TWF, respectively, allowed distinguishing between impaired and intact human skin samples in general. Single skin samples did, however, not, poorly and even inversely correlate with the test-compound absorption. In contrast, results with ISTD (e.g. ^{3}H-testosterone) were highly correlated to the absorption of ^{14}C-labeled test compounds. Importantly, ISTD did not influence analytics or absorption of test compounds. Therefore, ISTD, especially when adjusted to the physico-chemical properties of test compounds, is a promising concept to assess the integrity of skin samples during the whole course of absorption experiments. However, a historical control dataset is yet necessary for a potential routine application.

1. Introduction

For compounds which may get in contact with the skin, knowledge of dermal absorption is necessary to estimate the systemic exposure and perform risk assessments. For the determination of the systemic available amount of a compound in contact with the skin in vivo, in vitro and *in silico* methods are established (Schäfer and Redelmeier, 1996b). The in vitro method outlined in the OECD test guideline no. 428 is accepted by many regulatory agencies and is in accordance with the aim to reduce animal testing (OECD, 2004a, 2004b). Excised human or animal skin is mounted on a diffusion chamber, test compound is applied topically and the penetrated and permeated amount is measured in the skin sample and the underlying receptor fluid. The protocol was subject of multicenter validation studies as laid down (van de Sandt et al., 2004) and following specifications of e.g. skin type and handling (Schäfer-Korting et al., 2006, 2008). To avoid unsuitable over-prediction of the dermal absorption by the use of impaired skin preparations, the OECD guideline requires a skin integrity check. This test should ensure the exclusive use of data generated with skin with intact barrier function. In addition to a visual examination of the skin, the guideline proposes measuring the TEER (transepidermal electrical resistance), TEWL (transepidermal water loss) or the absorption characteristics of a reference compound in advance or at the end of an experiment, e.g. ^{3}H-water (TWF, transepidermal water flux), or concurrently by adding an internal reference standard (ISTD) with high specific activity to the test compound preparation, e.g. ^{3}H-sucrose (OECD, 2004a, 2004b).

Widely used standard methods in many laboratories are TWF and TEWL and TEER (Diembeck et al., 1999; Meidan and Roper, 2008). Despite intensive investigations, there is an ongoing debate about experimental performances, limit values and fields of

Abbreviations: AD, potentially absorbable dose; BLUE, methylene blue absorption; BSA, bovine serum albumin; CV, variation coefficient; DMA, dimethylamine; EDTA, ethylenediaminetetraacetic acid; ISTD, internal reference standard as well as integrity test using an internal reference standard; Kp, permeability coefficient (infinite dosing); logP, logarithmic octanol/water partition coefficient; LSC, liquid scintillation counting; maxKp, maximal permeability coefficient (finite dosing); MCPA, 2-ethyl-4-chlorophenoxyacetic acid; MCPA-2EHE, 2-methyl-4-chlorophenoxyacetyl ethylhexylester; MW, molecular weight; R^2, correlation coefficient; SD, standard deviation; SRA, specific radioactivity; TEER, transepidermal electrical resistance; TEWL, transepidermal water loss; TWF, transepidermal water flux.

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application (Brain et al., 1995; Chilcott et al., 2002; Meidan and Roper, 2008; Netzlaff et al., 2006). For example, TWF is a widely used and established marker for skin barrier function with a large historical dataset (Bronaugh et al., 1986; Meidan and Roper, 2008). Yet, the application of an infinite dose of water and therefore hydration for several hours, followed by the necessary removal and wash, may cause physical deterioration of the skin and higher permeability afterwards (Brain et al., 1995) whereas TWF measurement at the end of the experiment may lead to rejection of previously intact skin samples. Because of most similar treatment of the skin this is conceivable for TEER (Davies et al., 2004; Fasano et al., 2002), too. Also, TEWL is widely used as a marker for skin barrier function in vitro and in vivo. While avoiding physical stress to the skin (Levin and Maibach, 2005), like TEER and TWF, TEWL provides only a snapshot before or after an experiment. However, vehicle ingredients can damage the stratum corneum structure and hydration level; deterioration with time has been reported (Buist et al., 2005; Shah et al., 2008). The same holds true for the integrity test BLUE which utilizes the absorption of methylene blue as a measure for barrier functionality.

In contrast to TWF, TEER, TEWL and BLUE the integrity test ISTD supplies information of the barrier function over the whole experimental period and avoids the elongation of the test period. But the presence of an additional compound in the donor may influence the absorption characteristic of the test compound because of changes in solubility or saturation levels of the test compound and effects of the solvent on the barrier system (Barry, 1987; Dugard and Scott, 1986). Due to this influence the inertness of an ISTD must be proven. ^{3}H-sucrose and phenol red have been used as ISTD in the past, but systematic validation and provision of a sufficient dataset is still missing (Balaguer et al., 2006; Pendlington et al., 1997; Walters et al., 1997).

The purpose of the current work was to investigate the suitability of different skin integrity tests to differentiate impaired and intact human skin. Based on the absorption results of four test compounds (testosterone, caffeine, 2-ethyl-4-chlorophenoxyacetic acid (MCPA) and 2-methyl-4-chlorophenoxyacetyl ethylhexylester (MCPA-EHE)) through human and generally more permeable reconstructed human skin (StrataTest®), the common limit values for the standard integrity methods TEER, TWF and TEWL were assessed. Additionally, results of five skin integrity tests (TEER, TWF, TEWL, ISTD and BLUE) were correlated to absorption results derived with human skin or reconstructed human skin to evaluate their ability to explain minor differences in barrier function. Full-thickness and dermatomed human skin samples were applied to check for a possible effect of the skin preparation. Due to a lower donor dependency, rat skin was used in addition and chosen for a special experiment in which skin samples were systematically damaged to different grades before use. As model ISTD ^{3}H-testosterone was chosen. It was applied in parallel to test compound ^{14}C-MCPA. For human skin experiments two further well-investigated reference compounds with different physico-chemical properties were applied as ISTDs (^{3}H-caffeine and ^{3}H-mannitol) (OECD, 2004a; Peck et al., 1995; Schäfer-Korting et al., 2008; van de Sandt et al., 2004) to get an insight on the effect of ISTD selection. Additional experiments were conducted to check for effects of the present ISTDs on the analytics and absorption characteristics of the test compound.

2. Materials and methods

2.1. Chemicals and reagents

MCPA-2EHE, MCPA, dimethylamine (DMA; 60%), silicone antifoam emulsion (SRE) and ethylenediaminetetraacetic acid (EDTA) were provided by AH Marks and Co, Wyke, Bradford, Great Britain. Testosterone, caffeine, ethanol and methylene blue were purchased from Sigma Aldrich, St. Louis, MO, USA, bovine serum albumin (BSA) was from Roche, Basel, Switzerland, Texapon® N70 from Cognis, Düsseldorf, Germany, NaCl from Merck, Darmstadt, Germany and Soluene 350® and scintillation cocktail Hionic Fluor™ from Perkin–Elmer, Boston, MA, USA. Radiolabeled compounds (radiochemical purity >97%) were supplied by American Radiolabeled Chemicals, St. Louis, MO, USA (^{3}H-caffeine with 2.22 TBq mmol^{-1}), Perkin–Elmer (^{14}C- and ^{3}H-testosterone with 2.1 GBq mmol^{-1} and 6.3 TBq mmol^{-1}, respectively, ^{14}C-caffeine with 1.89 GBq mmol^{-1} and ^{3}H-mannitol with 455.6 GBq mmol^{-1}, ^{3}H-Water with 37 MBq ml^{-1}) or by AH Marks and Co (^{14}C-MCPA with 1.88 GBq mmol^{-1} and ^{14}C-MCPA-2EHE with 1.02 GBq mmol^{-1}). The radioactive isotopes are generally located at stable positions of the molecule: ^{14}C in the A ring of the steroid testosterone, in phenyl ring of MCPA and MCPA-EHE and in the methyl group at N-1 of caffeine; ^{3}H generally at non-acidic groups (testosterone at positions C-1, C-2, C-6, C-7, C-16 and C-17, mannitol at C-1 and caffeine in methyl group at N-1).

2.2. Skin preparations

Split-thickness (450 ± 100 μm) and full-thickness (1000 ± 200 μm) female human skin samples from abdominal surgery were purchased from Biopredic, France. Rat skin was excised from the back of eight-week-old female Crl:WI (Han) rats (Charles River, Germany) after sedation with isoflurane and exsanguination. Split-thickness skin (450 ± 100 μm) was generated with a Dermatome GA 643 (Aesculap, Germany) after hair trimming. For a special investigation various grades of barrier impairment were induced by stressing excised rat skin with chemical or mechanical treatment in advance of experiments using ^{14}C-MCPA as the test substance. Such pretreatment scenarios comprises combinations of water application or application of MCPA formulation (see Table 1) with or without MCPA and one or three washing steps with cotton swabs and 0.7% aqueous Texapon® N70 solution over three consecutive days. The individual treatments are given in Table 2. Experiments 1–3 comprise the 'undamaged' skin and experiments 4–9 the 'damaged' skin. StrataTest® (100–115 μm) purchased from Stratatech Corporation, USA, is a reconstructed human skin model which was added in the current setup as a human skin system with generally lower barrier functionality.

2.3. In vitro dermal absorption study

All studies were conducted following the OECD-Guideline 428 and the corresponding technical guidance document 28 (OECD, 2004a, 2004b). Five skin samples per run, derived from at least two different donors, were mounted on Franz type diffusion cells with a surface area of 1 cm^{2} and receptor volume of 4 ml (Laboratory Glass Apparatus Inc., USA). The water jacket around the receptor compartment was maintained using a water thermostat pump (Thermo Haake, Germany) at a temperature of 32 °C. A finite dose was applied to the surface of the skin under occlusive (Parafilm "M"®, Pechiney Plastic Packaging, USA) or semi-occlusive (Fixomull®, BSN medical, Germany) conditions. The receptor fluid was chosen to provide an adequate solubility of the test compound – at least 10 times higher than the maximal achievable concentration (see Tables 1 and 3). After exposure for 6 or 24 h the compound was washed off with cotton swabs and washing fluid. During the experimental period, samples were taken from the stirred (magnetic stirrers, Variomag Telemodul 20C/40C, H + P Labortechnik, Germany) receptor fluid at distinct time points and replaced with fresh receptor fluid by a fraction collector (222 L, Abimed, Germany) and a multi-channel peristaltic pump (MC 360, Ismatec, Germany). At the end of the run each diffusion cell was dismantled and all parts