Dermal absorption and hydrolysis of methylparaben in different vehicles through intact and damaged skin: Using a pig-ear model in vitro

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Currently, there is a trend to reduce of parabens use due to concern about the safety of their unmetabolised forms. This paper focused on dermal absorption rate and effectiveness of first-pass biotransformation of methylparaben (MP) under in-use conditions of skincare products. 24-h exposure of previously frozen intact and tape-stripped (20 strips) pig-ear skin to nine vehicles containing 0.1% MP (AD, applied dose of 10 μg/cm²), resulted in 2.0–5.8%AD and 2.9–7.6%AD of unmetabolised MP, and 37.0–73.0%AD and 56.0–95.0%AD of p-hydroxybenzoic acid, respectively, in the receptor fluid. The absorption rate of MP was higher from emulsions than from hydrogels, from enhancer-containing vehicles than from enhancer-free vehicles, and when skin was damaged. Experiments confirmed that the freezing of pig-ear skin slightly reduces hydrolysis of MP. After 4-h exposure of intact freshly excised and intact frozen skin, amount of <LOQ-2.3%AD and 2.3–3.3%AD unmetabolised MP, respectively, were found in the receptor fluid. Taking into account the number of useful properties of MP, but also the potential of systemic availability of unmetabolised MP, we consider that MP is more suitable for preserving rinse-off topical products than for leave-on products. Risk of systemic absorption of parabens should also be explored via the skin with damaged barrier.

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1. Introduction

Roughly 70 years, the parabens, alkyl esters of p-hydroxybenzoic acid (PHBA), were considered to be substances having low toxicity and some other properties of ideal preservatives. Parabens possess a broad spectrum of antimicrobial activity and water/oil solubility, excellent stability over a wide pH range, and low price (Soni et al., 2002, 2005; CIR, 2008). Furthermore, products containing parabens can be autoclaved. Therefore, either alone or in combination with other preservatives, parabens are used in a wide range of cosmetic, pharmaceutical, and partially also food products.

In our market survey conducted in 2011–2012 in the Slovak Republic (SR), according to the ingredients listed on the label, among 430 evaluated leave-on cosmetics at least one paraben was found in 39% products. Parabens were most frequently present in emulsion such as body-care and sunscreen creams and milks (including products intended for baby), hydrogels, lotions, and makeups. Among 159 topical medications registered in the SR, at least one paraben was listed in 10% of prescription and 5% of over-the-counter drugs. Methylparaben (MP) was present in 98% and 88%, propylparaben (PP) in 67% and 50%, respectively of paraben-positive cosmetics and medicines, while ethylparaben (EP) and butylparaben (BP) were listed sporadically only (Hojerová et al., 2013).

At recent years, the safety of parabens has become questionable. However, studies investigating the health effects of parabens are conflicting. Using a wide variety of assay systems in vitro and in vivo, a large number studies have demonstrated, that parabens may affect human health due to their endocrine disrupting activity (Routledge et al., 1998; Byford et al., 2002; Lemini et al., 2003; Pugazhendhi et al., 2005; Akomeah et al., 2007; Prusakiewicz et al., 2007; Darbre and Harvey, 2008; Boberg et al., 2010; Vo et al., 2010, 2011; Hu et al., 2013). Other researchers (van Meuwen et al., 2008; Shaw and deCatanzaro, 2009; Witorsch and Thomas, 2010; Sciali, 2011; Aubert et al., 2012; Kirchhof and
de Gannes, 2013) have published a radical opposition to concerns about health risks from doses of parabens commonly used. In 2004 Darbre’s team (Darbre et al., 2004; Harvey and Darbre, 2004) firstly measured trace residues (nanograms/g of tissue) of intact parabens, particularly MP, in human breast cancer tissues, and suggested that their presence in the human body might originate from topical application of body-care cosmetics such as under-arm deodorants and antiperspirants. Although some public health authorities (SCCP, 2005a,b, 2011a,b; FDA, 2007) and some cancer experts (Gikas et al., 2004; Rageth, 2005; CIR, 2008; Namer et al., 2008) rejected these considerations, the study has sparked controversy and also stimulated new international research on parabens effects on human health.

The Food and Drug Administration (FDA) in the USA, as well as the European Scientific Committee on Consumer Safety (SCCS) re-opened the safety assessment for parabens, to request exposure estimates and risk assessment for cosmetic use. According to the opinion of the SCCS (2011a), parabens may really exert a weak estrogen-like activity but its potency is from 1000 (for MP) to 1,000,000 (for BP) times below the potency of the positive control 17β-estradiol. So the SCCS (2011a, 2013) considers that for general cosmetic products containing parabens, excluding specific products for the nappy area, there is no safety concern in children (any age group) and adult consumers. Regarding personal care products, FDA (2007) states parabens safe at concentrations up to 0.8% (mixtures of parabens) or up to 0.4% (single paraben). The SCCS recognises a mixture of parabens safe also at concentrations up to 0.4% (single paraben), but BP and PP up only to 0.15% individually or in combination (SCCS, 2011a,b, 2013).

However, general view on the safety of parabens is based on the assumption that the ester bonds in the parent compounds are quickly and nearly completely hydrolysed by carboxylesterases (EC 3.1.1.1) in the common metabolite, a non-specific PHBA (Soni et al., 2005; Boberg et al., 2010). Since PHBA is considered to be a compound without an endocrine effects (SCCS, 2011), carboxylesterases activity appears to be crucial for detoxification parabens. Several studies (Harville et al., 2007; Janjua et al., 2008; Boberg et al., 2010; Shirai et al., 2013) confirmed that orally administered parabens are indeed readily metabolised by carboxylesterases in the intestines and liver and then excreted without significant accumulation in the body. However, metabolism of parabensadministered dermally may be incomplete for some reasons. The main reason is a lower capacity of skin carboxylesterases compared to mammalian liver carboxylesterases (Harville et al., 2007; Prusakiewicz et al., 2007). Another reason may be the negative effect of skin esterase inhibitor (Bando et al., 1997; Seko et al., 1999; Prusakiewicz et al., 2007; Harville et al., 2007; Jewell et al., 2007a,b), long-term use of a wide range of paraben-positive topical preparations, as well as inter-individual variations of human skin (Darbre et al., 2004; Harvey and Darbre, 2004; Ishiwatari et al., 2007; Darbre and Harvey, 2008). So it is generally accepted that unmetabolised (intact) forms of parabens in the body tissues are more likely the result of dermal applications than oraladministration (Oh et al., 2002; Prusakiewicz et al., 2007; El Hussein et al., 2007; Harville et al., 2007; Janjua et al., 2007; Darbre and Harvey, 2008; Williams, 2008; Barr et al., 2012; Shirai et al., 2013).

Considerable number of studies through intact human and various animal skin in vivo and in vitro have documented many parameters influencing the overall dermal absorption rate of parabens, i.e. without detection of individual quantities of unmetabolised parabens and their metabolite, PHBA (Pozzo and Pastori, 1956; Kitagawa et al., 1997; Oh et al., 2002; El Hussein et al., 2007; Janjua et al., 2007, 2008; Jewell et al., 2007a; Mbah, 2007; Pedersen et al., 2007; Wilkinson et al., 2007; Caon et al., 2010; Romonchuk and Bunge, 2010). Unfortunately, the available studies on the degree of hydrolysis of parabens during percutaneous permeation are limited. Bando et al. (1997) reported that after application of BP and PP to the rat skin in vitro, about 4% of intact BP and about 30% of intact PP from the total permeants in the receptor fluid was detected. Ishiwatari et al. (2007) evaluated the influence of daily exposure to MP containing formulations to human skin. At 1 h after a single application of 0.15% MP in emulsion to the forearm of human volunteers, unmetabolised MP (unmMP) concentrations about 18% of the application quantity of the parent MP were found in the stratum corneum (SC), but after 12 h the concentration of unmMP was decreased at 10 pmol/cm² (approximately 0.028%). However, the authors confirmed that repeated application of MP containing topical products significantly increases the amount of unmMP in the SC. The same researchers studied also the metabolism of MP through Yucatan micropig skin in vitro. 2-h exposure to an aqueous solution (10 μg/cm²) containing 0.1% of MP resulted in 2.06 mgof unmMP and 0.36 mg of PHBA expressed to 1 g tissue of full-thickness skin (Ishiwatari et al., 2007). Aubert et al. (2012) measured the content of unmMP in excretes from rats following dermal application for 6 h at a dose of 100 mg/kg of MP. No parent ester, only metabolite PHBA in the urine and feces (14–27% and <2% of the applied dose, respectively) was determined.

According to Aubert et al. (2012) in line with our view, the pivotal question of the safety assessment of parabens-containing topical products is the fate after human skin exposure, (a) their dermal absorption rate and (b) whether they absorbed intact or after first-pass hydrolysis in the skin. So the first aim of this study was to assess systemic exposure of unmMP and its main metabolite PHBA as a result of single topical application of different MP-containing products to ex-vivo intact skin (frozen prior to the experiments). Because of anatomical, physiological and biochemical similarity to human skin (Sekkat et al., 2002; Singh et al., 2002; Godin and Touitou, 2007; Jacobi et al., 2007; Klang et al., 2012; Lau et al., 2012), excised pig-ear skin as a skin model was chosen.

When investigating dermal absorption values of substances, risk assessment is generally focused on intact skin. The OECD (2004), as well as the SCCS (2010) guidelines for studies on in vitro dermal absorption prescribe also optimal barrier integrity of the excised skin. Such membrane is suitable to predict dermal absorption values through intact skin but may not be relevant for situation, where the topical product is applied to barrier-impaired skin due to mechanical, physical, chemical or biological reasons. Several studies (Jacobi et al., 2007; Lademann et al., 2009; Weigmann et al., 2009; Klang et al., 2012, 2013) have shown that stripped ex-vivo pig-ear skin is very representative to the in vivo human skin with barrier impaired due to mechanical trauma. To our knowledge, skinbarrier damage due to stripping in relation to the permeation of parabens has not been studied yet. Therefore, the second aim of this study was to assess the same objectives as in the first aim, but through stripped skin (frozen prior to the experiments).

Conflicting reports concerning the effect of storage conditions of the skin on percutaneous absorption of chemicals and degree of hydrolysis of ester bonds are published. While several reports suggest that the freezing of the skin alters the permeability of certain compounds (Hadzija et al., 1992; Shaikh et al., 1996; Wester et al., 1998; Ahlstrom et al., 2007; Payne et al., 2013), Harrison et al. (1984) has shown that the permeability of human skin ex-vivo is not significantly affected after prolonged freezing at −20°C up to 466 days. Two studies have demonstrated reduced activity of non-specific esterases in the suspension of frozen stored (at −20°C) pig ear skin towards retinyl ascorbate (Abdulmajeed et al., 2006) and acetylsalicylate (Lau et al., 2012) compared to freshly excised pig-ear skin. Conversely, a number of studies have shown for snake, human, rat, rabbit, guinea-pig, and mouse skin, that...
esterase activity can be almost or absolutely completely preserved for frozen skin stored at −20 °C (Nghiem and Higuchi, 1988; He Witt et al., 2000; Beydoun et al., 2010), and even at −70 °C (Jewell et al., 2007a) and at −80 °C (Stinchcomb et al., 2002). So the third aim of this study was to clarify whether storage by freeze-drying of the pig-ear skin affects dermal absorption rate and hydrolysis degree of MP.

2. Materials and methods

2.1. Chemicals

MP and PHBA (Table 1), both analytical-grade of ≥99%, were supplied by Sigma–Aldrich Chemie (Deisenhofen, Germany). Ingredients for cosmetic vehicles were received from local cosmetic producers. Water, acetonitrile and methanol (all HPLC grade) were supplied from Fisher Scientific (Loughborough, UK); Transcutol® PG (CAS 111-90-0) from Gattefossé (Saint Priest, France), urea (CAS 57-13-6), propylene glycol (CAS 57-55-6), and all other chemicals from Mikrochem (Pezinok, Slovak Republic) were of reagent grade. The receptor fluid consisted from phosphate-buffered saline (pH 7.4, own preparation) and 0.01% of Gentamicin-sulphate (Lek-Sandoz, Ljubljana, Slovenia) added for stabilization of the pig membranes.

2.2. Vehicles with or without penetration enhancers

Because the most common paraben in our survey, for dermal absorption study methylparaben was used. Nine formulations, representing the most frequently types of MP-containing topical leave-on products, were prepared according to the composition showed in Table 2. Each formulation contained MP at the target concentration of 0.1% (w/w), i.e. within the range of its using (a) in the skin-care cosmetics (0.1%, El Hussein et al., 2007; 0.007–0.409%, Eriksson et al., 2008; 0.03–0.3%, our unpublished results), (b) in the topical medications (0.02–0.3%, Soni et al., 2002). Three emulsions oil-in-water and three non-alcoholic hydrogels contained also one of the three chemicals known as penetration enhancers; Transcutol® PG (CAS 111-90-0) from Gattefossé (Saint Priest, France), urea (CAS 57-13-6), propylene glycol (CAS 57-55-6), and all other chemicals from Mikrochem (Pezinok, Slovak Republic) were of reagent grade. The receptor fluid consisted from phosphate-buffered saline (pH 7.4, own preparation) and 0.01% of Gentamicin-sulphate (Lek-Sandoz, Ljubljana, Slovenia) added for stabilization of the pig membranes.

2.3. Preparation and storage of skin sheets

Fresh ears from 6 months old domestic pigs (Slovak large white) were obtained from a local abattoir immediately post-mortem and prior to steam cleaning. Following brief cleaning with tap water, the sheet of the full-thickness skin (FTS, consisting of the SC, viable epidermis, and dermis) was separated from the underlying cartilage on the upper half part of ear using a scalpel. Hairs were cropped to a length of 3 mm with an electric hair clipper. The FTS sheets with some visible imperfections of the SC, viable epidermis, and dermis) was separated from the underlying cartilage on the upper half part of ear using a scalpel. Hairs were cropped to a length of 3 mm with an electric hair clipper. The FTS sheets with some visible imperfections were excluded. For the experiments referred in the Section 2.6. All experiments were conducted under unoccluded and without an enhancer. For comparison a simple aqueous solution was prepared.

2.4. Tape stripping

The aim of this process was to mimic in vivo conditions of topical medications and cosmetic products containing parabens applied to human skin with the SC impaired due to various mechanical reasons. The previously frozen FTS sheets were blotted dry with a soft tissue and the thickness was measured at ten different locations of each of them using a micrometre (Digital micrometre SKW 1; Helios Mess-technik, Niedernhall, Germany; readability of 1 μm). Only sheets with thickness from 1.020 to 1.120 mm were used for further experiments. Subsequently, every second FTS sheet was stripped 20-times with the adhesive tape (3 M Scotch®) by the same person according to the protocol by Klang et al. (2012). Then the thickness of FTS sheet was measured again (Table 5). Distribution of corneocytes on 1st, 10th, and 20th tape-strips (Fig. 1a–c) was examined under the Leica® DM-1000 microscope and representative micrographs taken with a Leica® digital camera (Leica Microsystems, Wetzlar, Germany).

2.5. Diffusion apparatus

The experiments were performed according to the Guideline for the in vitro assessment of dermal absorption of cosmetic ingredients SCCS (2010). Pre-calibrated static unjacketed Franz-type diffusion cells (JM-Glass, Bratislava, SR) with a receptor chamber volume of 5.5 ± 1 mL and an area of 2.00 cm² available for diffusion were used. The FTS sheet was cut into three circular discs with a diameter of about 3.3 cm. The FTS disc was mounted in the diffusion cell with the stratum corneum towards the donor chamber and fixed with a metal clip. The receptor chambers were filled with degassed receptor fluid. Then the cells were immersed in a thermostatic water-bath to maintain the surface temperature of the FTS disc at 32 ± 1 °C throughout the experiment. The solution in the receptor chamber was continuously agitated at 600 rpm using a small Teflon-coated magnetic bar driven by a submersible magnetic stirrer (Variomag; Thermo Scientific, Karlsruhe, Germany). After equilibrating for 1 h, the barrier integrity of the FTS disc was checked.

2.6. Skin barrier integrity

The integrity of the skin, crucial for experiment was determined by transdermal electrical conductivity (TEC) across the FTS under the conditions of the method, as described in our previous papers (Klimová et al., 2012; Lucova et al., 2013). Only FTS discs that passed the TEC test (the TEC value of ≤1.0 mS/cm for intact FTS and 1.5–3.0 mS/cm for FTS after 20 strips) were used for further experiments (Table 3).

2.7. Formulation dosing

Aqueous solution (20 μL) was introduced into the donor chamber using an adjustable micropipette. For dosing emulsions and hydrogels, the cells were dosed. The FTS disc was blotted dry with a soft tissue and placed on an electronic balance. A few small drops of formulation were deposited on the exposure area, homogeneously spread and accurately weighed to a quantity of 20 ± 0.1 mg. The disc was then mounted in the same place of the diffusion cell and fixed with a metal clip. Attention was paid to remove air bubbles. The exact time of application was noted (time zero). Then diffusion cell was immersed in a water-bath under the conditions described in the Section 2.6. All experiments were conducted under unoccluded donor chambers since a vast majority of topical leave-on formulations is applied open to the atmosphere.

2.8. Experimental design

Two sets of experiments were carried out as follows. First set, concerning the fate of MP after exposure of previously frozen intact FTS and stripped FTS, consisted from two steps: (a) 24-h kinetic studies of MP and PHBA for nine formulations, and (b) 4-h studies of dermal absorption rate and degree of MP hydrolysis for nine formulations and the mass balance of MP for four emulsions. Second set of experiments concerning the effect of skin storage by freeze-drying of MP and degree of its hydrolysis, consisted from one step: 4-h studies of dermal absorption rate, degree of MP hydrolysis and mass balance of MP for four emulsions through freshly excised intact FTS and previously frozen intact FTS.

Table 1: Physicochemical characteristics of methylparaben and its metabolite, p-hydroxybenzoic acid.

<table>
<thead>
<tr>
<th>INCI name†</th>
<th>CAS#</th>
<th>CID#</th>
<th>MP#</th>
<th>MW# (g/mol)</th>
<th>Solubility† in water 25 °C (g/l)</th>
<th>Solubility† in methanol 25 °C (g/l)</th>
<th>Log Poc/w 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylparaben’</td>
<td>99-76-3</td>
<td>7456</td>
<td>C8H10O3</td>
<td>152.15</td>
<td>362.2</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>99-96-7</td>
<td>135</td>
<td>C6H5O2</td>
<td>138.12</td>
<td>6.0</td>
<td>1.56</td>
<td></td>
</tr>
</tbody>
</table>

† IUPAC name: Methyl 4-hydroxybenzoate (Pubchem, 2012).
‡ IUPAC name: 4-Hydroxybenzoic acid (Pubchem, 2012).
§ International Nomenclature of Cosmetic Ingredients (EC, 2006).
¶ Chemical Abstracts Service (Pubchem, 2012).
||(Compound) Identification Number – Chemical structure (Pubchem, 2012).
‡ Molecular Formula (Pubchem, 2012).
++ Molecular Weight (Pubchem, 2012).
* Jewell et al. (2007a).
* Josyba (2010).
% Partition coefficient n-octanol/water (Jewell et al., 2007a).
2.8.1. Studies through previously frozen intact skin and stripped skin

2.8.1.1. 24-h Kinetic studies.

Three FTS discs were obtained from one previously frozen intact FTS sheet, as well as stripped FTS sheet. To minimize experimental errors arising from possible variability of FTS quality, one disc for one of the three different formulations was used in the same experiment. Further procedures were carried out as described in the Sections 2.5–2.7. Formulation was left in contact with the skin for 24 h. At seven predetermined time intervals (1, 2, 3, 4, 5, 6, and 24 h), 50 µL of the receptor chamber content (abbreviated as RF) was sampled and replaced with the same volume of fresh RF. The sample was immediately assayed for a concentration of MP and PHBA via HPLC. The process was repeated two more times in other 2 days, so three cells with previously frozen intact FTS, as well as three cells with stripped FTS for each formulation were used. The same schedule of experiments was used for other six formulations.

2.8.1.2. 4-h Permeation rate and degree of hydrolysis.

Based on the results of kinetic studies (see Section 3.1 and Fig. 3), 4-h exposure of the FTS to nine formulations were chosen for further experiments. Nine diffusion cells exposed to nine different formulations were investigated in the same experiment. Formulation was left in contact with the skin for 4 h. For Hydrogels 1–4 and the aqueous solution, at the

Table 2
Composition of formulations containing methylparaben mimicking leave-on topical preparations without or with chemical enhancers.

<table>
<thead>
<tr>
<th>Ingredienta</th>
<th>Aqueous solution (% w/w)</th>
<th>Hydrogel (% w/w)</th>
<th>Emulsion oil-in-water (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 without E</td>
<td>2 with UR</td>
<td>3 with TC</td>
</tr>
<tr>
<td>Methylparaben</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Aqua</td>
<td>q.s. to 100</td>
<td>q.s. to 100</td>
<td>q.s. to 100</td>
</tr>
<tr>
<td>Urea</td>
<td>–</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>Ethoxydiglycolb</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Olea Europaea oil</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glyceryl stearate</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C12-14 Pareth-3</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Carbomerc</td>
<td>–</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sodium hydroyxide</td>
<td>–</td>
<td>to pH 5.5</td>
<td>to pH 5.5</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

E: enhancer; UR: urea; TC: Transcutol; PG: propylene glycol.

* Name by the International Nomenclature of Cosmetic Ingredients (EC, 2006).

b Commercial name Transcutol® C210.
c Commercial name Carbopol® 940.

Fig. 1. Distribution of corneocytes on the removed tape strips. (A) 1st tape strip, (B) 10th tape strip, and (C) 20th tape strip. Micrographs taken through the Leica DM1000 light microscope with a Leica digital camera driven by software Leica Application Suite V3 (both from Leica Microsystems, Wetzlar, Germany).
end of the exposure, only the RF was analysed. For emulsions 1–4, also the mass balance and the total recovery of MP were evaluated. This schedule was repeated five times in other days. So, 6 diffusion cells with previously frozen intact FTS, as well as 6 cells with stripped FTS for each formulation were used. After 4-h exposure to an emulsion, the skin surface was washed four times with 0.5 mL of methanol. The rinsing solutions were collected into a vial, vigorously shaken with a vortex for 10 min and centrifuged at 6000 rpm for 10 min. The supernatant was analysed via HPLC to evaluate the extent of MP absorption in the skin area and PHBA content. As an additional hydrolysis result, the rinsing solution was cut into very small pieces using a scissors and introduced into a vial containing 5 mL of methanol, and subsequently processed as the rinsing solutions, with the exception of the vortexing time (6 h). The quantities of MP and PHBA in the supernatant were determined via HPLC. The extraction method was verified in blank experiments by spiking the FTS discs with a known amount of MP and PHBA; the total recovery percentages were included in the interval 96–101%. The RF was analysed via HPLC. Finally, the total recovery was calculated.

3. Results

3.1. Results of the first set of experiments

3.1.1. 24-h Kinetic studies (Fig. 3, Tables 3 and 4)

The total amounts of PHBA and unnmMP after 24-h permeation through previously frozen intact and stripped FTS in the RF from nine vehicles are summarised in Table 3. The amount of MP and PHBA in the RF, which permeated the skin per unit surface area during 24 h, was plotted against time. Four extreme permeation profiles of them are shown in Fig. 3. Among hydrogels, the lowest permeation rate was observed for Hydrogel 1 (without E) and the highest for Hydrogel 3 (with TC), among emulsions the lowest permeation rate was found for Emulsion 1 (without E) and the absolutely highest rate for Emulsion 3 (with TC).

To illustrate, the permeability parameters for Emulsion 3 were determined (Table 4). The lag time and the steady state flux ($J_{ss}$) were calculated by linear regression from the plot using the TableCurve 2D software program for Windows. The permeability coefficient ($P$) was calculated according to

$$ P = \frac{J_{ss}}{C} $$

where $P$ is permeation coefficient (cm/h × $10^3$), $J_{ss}$ is Flux steady state ($\mu g/cm^2 \times h$), and $C$ is Concentration of the parent MP added to the donor compartment ($mg/cm^2$).

Since the concentration of PHBA ≥ LOQ in the RF was detectable first time after 4-h skin exposure to Hydrogel 1 (Fig. 3), for further experiments just this 4-h exposure was chosen.

3.1.2. 4-h Permeation rate and degree of hydrolysis (Fig. 4)

The concentrations of PHBA and unnmMP (expressed as %AD of the parent MP) measured in the RF after 4-h exposure of previously frozen intact and stripped FTS to nine vehicles were summarised in Fig. 4. Only negligible amounts (above LOD, below LOQ; marked as point B in Fig. 4) of unnmMP after permeation from the aqueous solution, Hydrogel 1, and Hydrogel 2 through both intact FTS and stripped FTS, as well as from Hydrogel 4 through intact skin were found. However, quantifiable amounts of MP from other vehicles were determined; 0.20 $\mu g/cm^2$ (i.e. 2.0%AD) of MP from Hydrogel
4 through stripped skin and 0.18 and 0.22 μg/cm² of MP from Hydrogel 3 through both intact skin and stripped skin, respectively. In the Section 3.1.3, the results concerning the dermal absorptions of MP from Emulsions 1–4 through previously frozen intact skin and stripped skin are given. In contrast to MP, after 4-h exposure of previously frozen intact FTS and stripped FTS from all nine formulations into the RF, considerable amounts of PHBA (27.0–72.6%AD expressed as MP) were determined. The stripping procedure resulted in significantly greater permeation rates of PHBA from all formulations tested relative to permeation rates through intact skin from the same formulation (Fig. 4).

### 3.1.3. Mass balance analysis for previously frozen intact skin and stripped skin (Table 5)

The total recovery of the parent MP was evaluated in 4-h permeation experiments for Emulsions 1–4 only. Concentration of PHBA and unmetabolised MP (μg/cm²) distributed in the compartments of the diffusion system for previously frozen intact FTS (6 plus 2

### Table 4

In vitro skin permeation parameters of unmetabolised methylparaben (MP) and its metabolite, p-hydroxybenzoic acid (PHBA) through previously frozen intact and stripped full-thickness pig-ear skin (FTS) from Emulsion 3 (10 ± 0.05 mg/cm², containing 10 ± 0.05 μg/cm² of MP).

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Permeant</th>
<th>Lag time (h)</th>
<th>Jₚ (μg/cm² h)</th>
<th>C (mg/cm²)</th>
<th>P (cm/h × 10⁵)</th>
<th>P (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact FTS</td>
<td>PHBA</td>
<td>N.d.</td>
<td>2.16</td>
<td>1.00</td>
<td>2.16</td>
<td>0.600</td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>1.00</td>
<td>0.13</td>
<td>1.00</td>
<td>0.13</td>
<td>0.036</td>
</tr>
<tr>
<td>Stripped FTS</td>
<td>PHBA</td>
<td>N.d.</td>
<td>2.52</td>
<td>1.00</td>
<td>2.52</td>
<td>0.700</td>
</tr>
<tr>
<td></td>
<td>MP</td>
<td>N.d.</td>
<td>0.18</td>
<td>1.00</td>
<td>0.18</td>
<td>0.050</td>
</tr>
</tbody>
</table>

N.d.: Not detectable; Jₚ: Steady state flux; C: Concentration of the parent MP (0.1% w/w, i.e. approx. 1 mg/cm³); P: Permeability coefficient.

Data are calculated from three replicates (Fig. 3).

### Table 5

Distribution of unmetabolised methylparaben (MP) and its metabolite, p-hydroxybenzoic acid (PHBA, expressed as MP) in a compartment of the diffusion system after 4-h exposure of intact freshly excised, intact frozen and stripped frozen stored (at −20 °C, maximum 6 weeks) full-thickness pig-ear skin (FTS) to each of the four emulsions containing 0.1% of MP (dose of MP 10 ± 0.05 μg/cm²).

<table>
<thead>
<tr>
<th>Membrane frequently excited FTS</th>
<th>Compartments of the diffusion system</th>
<th>Emulsion 1 without E</th>
<th>Emulsion 2 with UR</th>
<th>Emulsion 3 with TC</th>
<th>Emulsion 4 with PG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MP</td>
<td>PHBA</td>
<td>MP</td>
<td>PHBA</td>
<td>MP</td>
</tr>
<tr>
<td>Intact FTS</td>
<td>Surface (μg/cm²)</td>
<td>2.10 ± 0.23</td>
<td>&lt;LOD</td>
<td>2.01 ± 0.31</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td></td>
<td>Skin (μg/cm²)</td>
<td>1.19 ± 0.10</td>
<td>&lt;LOQ</td>
<td>1.13 ± 0.24</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>Receptor fluid (μg/cm²)</td>
<td>2.84 ± 0.10</td>
<td>4.06 ± 0.56</td>
<td>4.55 ± 0.46</td>
<td>3.9</td>
</tr>
<tr>
<td>Thickness 1.025–1.200 (mm)</td>
<td>MP from (MP + PHBA) in receptor fluid (%)</td>
<td>Incalculable</td>
<td>Incalculable</td>
<td>91.9</td>
<td>76.9</td>
</tr>
<tr>
<td></td>
<td>Total recovery (MP + PHBA) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact frozen stored FTS²</td>
<td>Surface (μg/cm²)</td>
<td>1.28 ± 0.33</td>
<td>&lt;LOD</td>
<td>1.26 ± 0.30</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td></td>
<td>Skin (μg/cm²)</td>
<td>2.41 ± 0.65</td>
<td>&lt;LOQ</td>
<td>2.33 ± 0.72</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>Receptor fluid (μg/cm²)</td>
<td>0.23 ± 0.05</td>
<td>4.58 ± 0.39</td>
<td>4.66 ± 0.53</td>
<td>3.33 ± 0.09</td>
</tr>
<tr>
<td>Thickness 1.020–1.195 (mm)</td>
<td>MP from (MP + PHBA) in receptor fluid (%)</td>
<td>4.8</td>
<td>4.7</td>
<td>5.1</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Total recovery (MP + PHBA) (%)</td>
<td>85.0</td>
<td>84.8</td>
<td>91.5</td>
<td>86.0</td>
</tr>
<tr>
<td>Stripped frozen stored FTS³</td>
<td>Surface (μg/cm²)</td>
<td>1.08 ± 0.22</td>
<td>&lt;LOD</td>
<td>0.98 ± 0.18</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td></td>
<td>Skin (μg/cm²)</td>
<td>1.32 ± 0.48</td>
<td>&lt;LOQ</td>
<td>1.02 ± 0.14</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>Receptor fluid (μg/cm²)</td>
<td>0.32 ± 0.08</td>
<td>6.10 ± 0.91</td>
<td>6.52 ± 0.42</td>
<td>5.55 ± 0.15</td>
</tr>
<tr>
<td>Thickness 1.000–1.160 (mm)</td>
<td>MP from (MP + PHBA) in receptor fluid (%)</td>
<td>5.0</td>
<td>5.4</td>
<td>7.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Total recovery (MP + PHBA) (%)</td>
<td>88.2</td>
<td>88.9</td>
<td>98.8</td>
<td>91.3</td>
</tr>
</tbody>
</table>

E: enhancer; UR: urea (3%, w/w); TC: Transcutol® C9 (5%, w/w); PG: propylene glycol (5%, w/w).

<LOD: below the limit of detection; LOD for MP: 0.020 μg/mL; LOD for PHBA: 0.330 μg/mL (see Section 2.9).

<LOQ: below the limit of quantification.

LOQ for MP: 0.066 μg/mL, i.e. 0.066 μg/cm² on surface; 0.165 μg/cm² in skin; 0.182 μg/cm² in receptor fluid (see Section 3).

LOQ for PHBA: 0.090 μg/mL, i.e. LOD for PHBA: 1.089 μg/cm² on surface; 2.475 μg/cm² in skin; 2.995 μg/cm² in receptor fluid (see Section 3).

* Denotes the amount of MP and PHBA permeated through intact frozen stored FTS significantly different (p < 0.05) from the amount of the same compound through intact freshly excised FTS in a given compartment of the diffusion system.

* Denotes the amount of MP and PHBA permeated through stripped frozen stored FTS significantly different (p < 0.05) from the amount of the same compound through intact frozen stored FTS in a given compartment of the diffusion system.

* Denotes the amount of MP and PHBA permeated through stripped frozen stored FTS significantly different (p < 0.05) from the amount of the same compound through intact frozen stored FTS in a given compartment of the diffusion system.

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replicates) and stripped FTS (6 replicates) are reported in Table 5. As expected, the main compound in the RF, PHBA was found; 45.8–60.9%AD for previously frozen intact FTS and 61.0–72.6%AD for stripped FTS. In contrast to PHBA, only small quantities of MP (2.3–3.3%AD and 3.2–5.5%AD) in the RF after passage through previously frozen intact FTS and stripped FTS, respectively were found. A considerable amount of the parent MP remained on the surface of intact previously frozen FTS (10.7–12.8%AD) and stripped FTS (9.8–10.8%AD) discs. If certain amount of PHBA on the surface of both intact and stripped FTS was present, it was <LOD in all emulsions. Compared with the amount of PHBA remaining on the skin, even greater quantities of MP were accumulated in previously frozen intact FTS (10.7–12.8%AD) and stripped FTS (9.8–10.8%AD) discs. As freshly excised FTS for four different emulsions oil-in-water. According to the results summarised in Table 5, freezing of pig-ear skin only slightly reduces the hydrolytic activity of esterases. This result is in an agreement with the observation by Abra et al. (2006) that pig-ear skin stored frozen at –20°C for up to 2 months is still able to verify hydrolysis of an ester produg in the skin. Compared to intact previously frozen FTS in our study, the amount of unMP permeated in the RF through intact freshly excised FTS was not statistically significant for Emulsion 3 with TC and Emulsion 4 with PG, but was significantly lower (p < 0.05) for Emulsion 1 without enhancer and Emulsion 2 with UR. Insufficient total recovery for Emulsion 2 with UR (76.9%) and Emulsion 4 with PG (83.5%) after exposure of intact freshly excised FTS, than recommended by SCCS (2012) was probably due to the relatively large number of PHBA absorbed in the skin, but still lower than LOD. Our results about percentages from the applied dose of unMP in the RF (2.1–3.3%) for fresh, as well as previously frozen intact skin are in accordance with the SCCS (2011), which the 3.7% dermal absorption value of unmetabolised paraben uses in final calculations of the Margin of Safety (MoS).
Significantly decreasing amounts of corneocytes with an increasing tape number after tape-stripping of FTS sheets are documented in Fig. 1a–c. Although the skin damage by stripping in our experiment was more drastic than a normal skin mechanical damage in everyday life. However, we assume that due to repeated depilation, shaving, or skin injured for various reasons, the process is close to skin conditions in some consumers and patients.

Compared to intact FTS sheet, the stripping procedure caused only a slight decrease in the thickness of FTS sheet (on average of 11 ± 3 μm, i.e. approx. of 1%) but dramatically higher TEC value (on average of 11 ± 3 μm, i.e. approx. of 1%). Latest fact resulted in higher permeation rate of the parent MP and its metabolite from all nine formulations tested (Tables 3 and 5). After 24-h, there were found 2.9–7.6% AD of unmetabolised MP and its metabolite, p-hydroxybenzoic acid (PHBA) found in the receptor fluid (RF) from Hydrogel 1 and Emulsion 1 (both without enhancer) showed the lowest dermal absorption rate and Hydrogel 3 and Emulsion 3 (both with Transcutol® CG) showed the highest dermal absorption rate. Values are the mean ± SD (n = 3). B: below the limit of quantification (LOQ), over the limit of detection (LOD); LOD < B < LOQ.

Fig. 3. Absorption-time profiles of methylparaben (10 ± 0.05 μg/cm²) during the 24-h exposure of previously frozen intact and stripped full-thickness pig-ear skin (FTS). The amount (μg/cm²) of unmetabolised MP and its metabolite, p-hydroxybenzoic acid (PHBA) found in the receptor fluid (RF) from Hydrogel 1 and Emulsion 1 (both without enhancer) showed the lowest dermal absorption rate and Hydrogel 3 and Emulsion 3 (both with Transcutol® CG) showed the highest dermal absorption rate. Values are the mean ± SD (n = 3). B: below the limit of quantification (LOQ), over the limit of detection (LOD); LOD < B < LOQ.
to 1.7-fold, respectively, through intact FTS and from 1.8 to 2.2-fold and 1.4 to 1.5-fold, respectively, through stripped FTS. This finding is consistent with the assumption that the more a substance is soluble in the vehicle, the more likely it will be transported (Baker, 1986). As shown in the octanol/water partition coefficient (Log \( K_{ow} \)) of MP (1.96, Table 1), the nature of the hydrophilic–lipophilic MP is closer to emulsion o/w than to high hydrophilic hydrogel and aqueous solution. In accordance with the results of the study by Watrobska-Swietlikowska and Sznitowska (2006) we assume that a high content of the parent MP was accumulated in the interface of the emulsion. Our results are in an agreement with the observation by Esposito et al. (2003) that the permeability coefficient of parabens increases with paraben water solubility for emulsions, whereas it decreases in the case of gels. The permeation rates of MP from emulsions were greater also probably due to the physical form of an emulsion is closer to the natural skin film as a form of a hydrogel or a solution. The \( J_{ss} \) values of PHBA and MP obtained in our study for Emulsion 3 were 2.16 and 0.13 \( \mu g/cm^2 h \) through intact skin and 2.52 and 0.18 \( \mu g/cm^2 h \) through stripped skin. Esposito et al. (2003) investigated the diffusion of MP in topical products containing 0.05% of MP through a sandwich of two membranes; (a) polydimethylsiloxane-based membrane and (b) a nylon-based membrane (150 \( \mu m \)) with 0.22 \( \mu m \) pore size. The researchers determined the \( J_{ss} \) value of MP (total amount of MP and PHBA) at 4.87 \( \mu g/cm^2 h \) from o/w emulsion and 3.95 \( \mu g/cm^2 h \) from hydrogel. Caon et al. (2010) in the 6-h kinetic study through intact full-thickness pig-ear skin exposed to 0.1% parabens (in an ethanol/PBS mixture 50:50) determined the \( J_{ss} \) value of MP of 20 \( \mu g/cm^2 h \) (total quantities of MP and PHBA). Unfortunately, the two above-mentioned groups of researchers did not assess metabolism of the parent MP.

The results of our study clearly showed that the addition of penetration enhancers to a formulation increases the amount of MP and PHBA in the RF and therefore also a potential their entry into the bloodstream. Transcutol \(^\text{\textregistered}\) CG was seen to have greater ability to enhance epidermal-dermal diffusivity of MP than vehicles with UR and PG. After 24-h passage, the amount of MP and PHBA from TC-containing Emulsion 3 through intact skin was 1.7 and 1.3-fold greater than through stripped skin 1.5 and 1.2-fold greater, respectively than from Emulsion 1 without enhancer. We consider that TC as solvent was acting by fluidizing lipids within the SC that allowed greater solute movement within the epidermal membrane. Due to this fact, when topical leave-on products preserved with MP are formulating, producer should avoid the use of penetration enhancers, except when it is necessary. When examined the proportion of MP from total flux of permeants in the RF (sum of MP and PHBA; see Table 4), the findings are notable. Regardless of the skin condition, the proportion of MP was approximately the same for certain formulation and ranged from 4.9% (for the aqueous solution) to 7.4% (for Emulsion 3).

Finally, despite the possible differences between the human skin and previously frozen pig-ear skin in the dermal rate and effectiveness of hydrolysis of MP, we calculated the Systemic Exposure Dosage (SED) of both MP and PHBA for humans treated with the finished cosmetic product. The SED value is the amount of a cosmetic ingredient expected to enter the blood stream (and therefore be systematically available) per kg body weight and per day (SCCS, 2012) and calculated according to

\[
\text{SED} = \frac{DA (\mu g/cm^2) \times SSA (cm^2) \times F (day^{-1})}{60 (kg)}
\]

where SED (\( \mu g/kg bw/day \)) is the Systemic Exposure Dosage; DA is dermal absorption of the substance reported as amount (\( \mu g/cm^2 \)); SSA (\( cm^2 \)) is the skin surface area expected to be treated with the cosmetic product; F (day\(^{-1}\)) is the usual frequency of application of the cosmetic product; 60 kg is default human body weight (SCCS, 2012).

In a classical in vitro dermal absorption setting, the total amount measured in the epidermis (without the SC), dermis and the receptor fluid is considered to be systematically available and taken into account for further calculations (SCCS, 2010, 2012; OECD, 2011). As mentioned above, after our experiments we were unable to separate quantitatively the SC from the rest of the FTS disc, both intact and stripped skin, mainly due the hair follicles. Also OECD GD 28 (OECD, 2011) states that fractionation of the skin after experiments can be difficult in some cases. However, if we hypothesize that a majority of the SC was removed as the consequence of the tape-stripping procedure (20-times), the amount of MP and PHBA found in stripped skin, was absorbed mainly in its living epidermal-dermal layers. Seko et al. (1999) studied the effect of cutaneous metabolism of PP and BP using intact and stripped rat skin in vitro. Consistent with our hypothesis, they also considered the amount of PP and BP found in the skin after tape-stripping procedure (15 times) as absorbed in the living layers of the skin.

Therefore, in our calculations, the SED values were evaluated for permeation only through stripped skin and under the two case scenarios. In a best case scenario, only the amount of MP in the RF after 4-h exposure was considered to be systematically available.
and used in the exposure calculations. In a worst case scenario, the total amount of MP (in the FTS disc and the RF) after 4-h exposure was considered to be systemically available and used in the exposure calculations. The same scenarios were used for PHBA.

Since propylene glycol is widely used in many types of cosmetic products including emulsions, Emulsion 4 with PG was selected as an illustrative example for the calculation of SED values for MP and PHBA after 4-h exposure of mechanically damaged skin (Table 6). Here we discuss two extremes among them. First, assuming that, a body milk with 5% PG containing 0.1% MP is applied to the female skin in vivo to a leave-on emulsion oil-in-water (10 mg/cm²) containing 0.1% of MP and 5% of propylene glycol (PG).

5. Conclusions

In this paper, the potential for systemic absorption of methylparaben through intact skin and mechanically damaged skin was studied. Four major findings can be drawn. Firstly, after a single skin exposure to MP containing products similar to the formulations tested, MP is not hydrolysed completely and certain amount of unhydrolysed methylparaben (MP) and its metabolite, p-hydroxybenzoic acid (PHBA), after 4-h exposure of damaged human skin in vivo to a leave-on emulsion oil-in-water (10 mg/cm²) containing 0.1% of MP and 5% of propylene glycol (PG). Here we discuss two extremes among them. First, assuming that a body milk with 5% PG containing 0.1% MP is applied to the female skin in vivo to a leave-on emulsion oil-in-water (10 mg/cm²) containing 0.1% of MP and 5% of propylene glycol (PG). In the best case scenario the amount of 250 and 3930 g/kg bw/day, in the worst case scenario 922.97 and 3930 g/kg bw/day, of unmMP and PHBA, respectively, become systemically available.

The authors declare that there are no conflicts of interest.

References


