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Skin permeation and metabolism of di(2-ethylhexyl) phthalate (DEHP)

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Abstract

Phthalates are suspected to be endocrine disruptors. Di(2-ethylhexyl) phthalate (DEHP) is assumed to have low dermal absorption; however, previous in vitro skin permeation studies have shown large permeation differences. Our aims were to determine DEHP permeation parameters and assess extent of skin DEHP metabolism among workers highly exposed to these lipophilic, low volatile substances.

Surgically removed skin from patients undergoing abdominoplasty was immediately dermatomed (800 μm) and mounted on flow-through diffusion cells (1.77 cm2) operating at 32°C with cell culture media (aqueous solution) as the reservoir liquid. The cells were dosed either with neat DEHP or emulsified in aqueous solution (166 μg/ml). Samples were analyzed by HPLC-MS/MS.

DEHP permeated human viable skin only as the metabolite MEHP (100%) after 8 hours of exposure. Human skin was able to further oxidize MEHP to 5-oxo-MEHP. Neat DEHP applied to the skin hardly permeated skin while the aqueous solution readily permeated skin measured in both cases as concentration of MEHP in the receptor liquid.

DEHP pass through human skin, detected as MEHP only when emulsified in aqueous solution, and to a far lesser degree when applied neat to the skin. Using results from older in vitro skin permeation studies with non-viable skin may underestimate skin exposures. Our results are in overall agreement with newer phthalate skin permeation studies.

Keywords: Di(2-ethylhexyl) phthalate, DEHP, 117-81-7, human, skin, percutaneous permeation

List of abbreviations:

2cx-MMHP: mono(2-(carboxymethyl)hexyl) phthalate
5OH-MEHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate
5oxo-MEHP: mono-(2-ethyl-5-oxohexyl) phthalate
5cx-MEPP: mono-(2-ethyl-5-carboxypentyl) phthalate
BSA: bovine serum albumin
CV: coefficients of variation
DAD: dermally absorbed dose
DEHP: diethyl hexyl phthalate
DiNP: diisononyl phthalate
DiDP: diisodecyl phthalate
DBP: di-butyl phthalate
EFSA: European Food Safety Authority
EPA: US Environmental Protection Agency
ESI: electrospray interface
EU: European Union
GHS: Globally Harmonized System of Classification and Labelling of Chemicals
HGP: hairless guinea pig
HMWP: High-molecular-weight phthalates
HPLC: a high-performance liquid chromatograph
τ : lag time
MBP: mono-butyl phthalate
MEHP: mono-(2-ethylhexyl) phthalate
J : permeation rate
Kp: permeation coefficient
OECD: Organisation for Economic Co-operation and Development
SC: stratum corneum
TDI: Tolerable Daily Intake
TEWL: trans epidermal water loss

1.0 Introduction

Phthalates are used to impart flexibility to plastics (plasticizers) and for their solvent properties in a wide variety of products (Graham, 1973). High-molecular-weight phthalates (HMWP) including diethyl hexyl phthalate (DEHP), diisononyl phthalate (DiNP), and diisodecyl phthalate (DiDP) are used in plastic tubing, food packaging and processing materials, containers, vinyl toys, vinyl floor coverings, and building products (Kueseng et al., 2007;ATSDR, 1990; ECB, 2008).

Phthalate exposures have produced a variety of male reproductive effects in animal studies (classified in the EU as category 2 substances to reproduction and as 1B in the GHS based classification system) (Skakkebaek et al., 2001). Phthalates may be absorbed into the body after ingestion, inhalation, and dermal exposures. The proportional contribution from each route of exposures is not known. Highly lipophilic substances such as phthalates are assumed to have low dermal absorption; however, few dermal absorption studies have included the skin's possibility to metabolize phthalates. Skin permeation rates used in dermal exposure assessments might therefore underestimate the absorbed dose in health risk assessments.

Human phthalate exposure is usually assessed by measuring urinary metabolites as the proportional contribution from the various sources and routes of exposure to phthalates is unknown. Phthalates are rapidly metabolized and excreted in urine and faeces after oral administration (ATSDR, 1990). An initial de-estrification of one alkyl linkage to the corresponding monoesters (one carboxylic acid and one ester substituent) is followed by an enzymatic oxidation of the alkyl chain, to more hydrophilic, oxidative metabolites after oral administration (Hauser and Calafat, 2005). For diethylhexyl phthalate (DEHP), the resulting monoester, mono-(2-ethylhexyl) phthalate (MEHP) is further oxidized, mainly by ω-oxidation at the terminal carbon of the alkyl ester side chain, to: mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP), and mono[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP) . The 5cx-MEPP is the primary metabolite (greater than 25%) in humans (Wittassek and Angerer, 2008), and MEHP is detected in typically less than 10% of the absorbed dose in the general population (Barr et al., 2003; Koch et al., 2003). After 24 hours post oral administration

to human volunteers, the urinary excretion for the sum of these five metabolites was between 65 and 70%, and half lives were between 2 and 24 hours (Koch and Angerer, 2007; Koch et al., 2005).

Workers manufacturing products containing phthalates are highly exposed and have shown to have urinary metabolite concentrations that often exceed those at the 95th percentile of the general population (NRC 2008). HMWPs are not very volatile, but they readily form aerosols that may be inhaled especially during work with heated processes (Smith et al., 1980). Dermal exposures could potentially play a role in non-heated processes; however, due to their lipophilicity dermal phthalate absorption is assumed to be low or negligible. This assumption might be false as several studies among HMWP exposed workers performing non-heated processes (Gaudin et al., 2011; Gaudin et al., 2008; Hines et al., 2008; Hines et al., 2009; Koch et al., 2012) have demonstrated the presence of urinary HMWP metabolites.

Gaudin *et al.* (Gaudin et al., 2011; Gaudin et al., 2008) found elevated urinary 5cx-MEPP metabolite concentrations (median 107.5 μg/L) among workers preparing DEHP-containing plastisol at room temperature and supervising the plastisol coating of glass flasks automatically dipped into vats filled with the plastisol. Moreover, Hines *et al.* (Hines et al., 2008) found elevated urinary 5OH-MEHP and 5oxo-MEHP (44.0 and 34.3 μl/L, respectively) among workers manufacturing vehicle filters with DEHP-based plastisol. In a very recent study (Koch et al., 2012); plastisol workers refinishing phthalate-containing sealants at room temperatures had 20 times higher DiNP/DiDP metabolite concentrations than the control group. Koch *et al.* (Koch et al., 2012) also found pre-shift urine samples to contain elevated phthalate metabolite concentrations, and the metabolite distribution was significantly different from oral studies. Two possible explanations were proposed by the authors: “(1) the maximum elimination was not achieved directly after the shift; or (2) skin metabolism might produce slightly different metabolite distribution than after oral exposure.”. Differences in metabolism may have potential implications for risk estimates. Therefore, improved knowledge concerning the dermal metabolism may provide better risk estimates of phthalate toxicity.

Human skin permeation may be efficiently evaluated using *in vitro* flow-through diffusion cells with fresh (metabolically active) excised skin. In this system the skin is isolated with no metabolic interference from enzymes in other parts of the body (Bronaugh, 2005). Flow-through diffusion (Bronaugh et al., 1982) cells are characterized by a continuously replaced receptor fluid, which represents to some extent *in vivo* conditions. This system is in particular suitable for phthalates which have very low solubility in the water based receptor fluid, increasing the sink conditions by continually replacing the receptor fluid (Brain et al., 1998). The primary barrier to skin absorption is often the nonliving stratum corneum (SC) layer on the surface of the skin, and metabolism occurs after SC permeation. For compounds that are insoluble in water and very soluble in oil such as for DEHP, the primary barrier to permeation shifts from the SC to the aqueous viable epidermis (Elsisi et al., 1989). The activity of skin soluble enzymes such as esterases, acetyltransferease, and alcohol and aldehydes dehydrogenases have been shown to substantially metabolize substances applied to viable skin in diffusion cells (Bronaugh, 2005). OECD (Guideline 427) recommends using human viable skin, although few skin permeation studies do, probably due to limited access.

Previous *in vitro* studies have shown large differences for DEHP permeation (Table 1), which could partially be explained by the difference in methods, especially the use of animal or non-viable skin. Our study is a first attempt to measure the DEHP permeation rate through excised human viable skin in the flow-through diffusion cell system. Specifically, our aims were: 1) Determine DEHP lag time (τ), permeation rate (J) and permeation coefficient (Kp); 2) Assess extent of DEHP skin metabolism; and 3) Estimate skin absorption in a simple occupational exposure task using the obtained permeation parameters.

2.0 Materials and Methods

2.1 Chemicals and materials

DEHP (CAS no. 117-81-7, molecular weight 390.6 g/mol, water solubility 41 μg/L at 25C (Leyder and Boulanger 1983), pKow 4.2-5.11, density 0.984 g/mL, vapour pressure 6.2×10^{-8} mm Hg at 25 °C) (99.5% pure) was obtained from Sigma-Aldrich (Buchs, Switzerland), while deuterated DEHP (d4-DEHP) was obtained from Cambridge Isotope Laboratories (ReseaChem GmbH, Burgdorf, Switzerland). MEHP (99.5% pure) was obtained from AccuStandard Europe

(Niederbipp, Switzerland). The cell culture media, RPMI-1640 Medium HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) Modification (R5886) was purchased from Sigma-Aldrich (Buchs, Switzerland).

2.2 Human skin

Surgically removed skin from patients undergoing abdominoplasty at the Department of Plastic and Reconstructive Surgery at the Cantonal University Hospital (Centre Hospitalier Universitaire Vaudois) in Lausanne, Switzerland, was collected immediately after removal. This skin still attached to its subcutaneous fatty layer, was brought to our laboratory in an ice-box and dermatomed. No more than 2 hours elapsed from time of surgery-end to the skin was placed in the flow-through diffusion cells. The surgeon obtained patient consent for the use of the removed skin in our experiments after human ethics approval. No personal information regarding the patients (gender, age, ethnicity, BMI, etc) was retained.

2.3 Flow-through diffusion cell experiments

A rack of six jacketed flow-through diffusion cells (receptor volume 8.5mL; 11.28 mm diameter opening; PermaGear bought from SES Analytical System, Bechenheim, Germany) was operated at 32°C. A fraction collector (FC 204, Gilson Inc., Middleton, WI, USA) automatically sampled the reservoir liquid. The peristaltic pump (Ismatec IPC-N, IDEX Health & Science GmbH, Wertheim-Mondfeld, Germany) was set at 40 µl/min, and replaced the collected reservoir liquid. The exposed skin area was 1.77 cm^2. Before each experiment, the system was rinsed with ethanol overnight followed by 2-3 hours of rinse with the receptor fluid prior to experiment to evacuate ethanol from the system. The skin was dermatomed (Acculan®II, B. Braun/Aesculap, Sempach, Switzerland) to a thickness of 800 µm. The six skin discs were mounted onto the diffusion cells filled with buffer solution (cell culture media), and the pump was calibrated after the skin had stabilized (approximately 30 min) determined by stable trans epidermal water loss (TEWL) (VapoMeter wireless, Delfin Technologies Ltd., Kuopio, Finland) readings. If the TEWL reading was above 11 $g/m^2/h$ (Pinnagoda et al., 1990), the skin disc was deemed damaged and replaced. The cells were dosed either with neat DEHP (2 ml) representing exposures among workers manufacturing DEHP or emulsified in buffer solution (d4-DEHP [166 µg/ml], MEHP [166 µg/ml]; 1.5 ml) representing aerosol exposures (the system does not allow the generation of

aerosols depositing directly on the skin disks). DEHP applied doses per cm^2 of skin were calculated to be 1,114.1 mg/cm^2 for neat and 140.7 mg/cm^2 for emulsified DEHP, respectively. The automated sampling collection was set for intervals lasting either 24 or 72 hours. Samples were collected in DEHP free vials.

2.4 Chemical analysis

The collection vials from the flow-through diffusion cell experiments were frozen at -20°C and shipped to the laboratory (the Institut National de Recherche Scientifique (INRS), France) for analysis under dry-ice and arrived frozen. Analyses of DEHP and its metabolites have been described previously, and only slightly modified (Gaudin *et al.*, 2008). Briefly, samples were injected into a high-performance liquid chromatograph (HPLC) with an on-line extraction on a restricted access material (Lichrospher RP-8 ADS from Merck-Darmstadt, Germany), followed by an analytical separation on a column (Phenomenex-Synergi 4 lm Polar-RP column; 80 Å, 150x25 mm).The analytes were then detected and quantified by MS/MS (1200 L Varian®) after introduction into an electrospray interface (ESI) in negative mode. The coefficients of variation (CVs) of the precision (intra- and inter-days) were <5%% for the three metabolites at any level (5, 10, 20, 100, 200 g/l). The quantification limits were 0.5 for MEHP, 5OH-MEHP, and 5-oxo-MEHP.

2.5 Data analysis

Permeability coefficients (Kp) were estimated from the slopes of the cumulative absorption plots over time. Lag times (τ) were estimated as the intercept of the steady state portion of the permeability rate (*J*) curves with the time axis. Individual Js were calculated from each diffusion cell and the average and standard deviations were calculated for the group. The Kp were estimated by dividing *J* with the density of chemical (DEHP as it was used neat) or concentration (d_4-DEHP and MEHP). Our study complies with the OECD guideline 428 in describing the skin origin and preparation and the proof of skin integrity using the TEWL, temperature (32°C), the choice of a suitable receptor fluid, the description of the diffusion cells used, the actual area of skin dosed, the number of cells/samples and donors (n=4), the duration of sampling period (24h), but does not include the determination of the amount retained in skin after washing.

3.0 Calculation of dermally absorbed dose

Skin absorption dose in a reasonable but hypothetical occupational exposure task: Due to maintenance reasons the vat containing the DEHP must be opened once per day, 200 times per year, and both hands will be immersed (800 cm^2) for about 3 minutes in the solution: (a) DEHP in aqueous solution with a concentration of 166 µl/ml (b) neat DEHP. The obtained permeation coefficient (Kp) was used in to calculate dermally absorbed dose (DAD) in mg/kg/day (EPA 1992).

$$DAD = \frac{DAevent * EV * ED * EF * A}{BW * AT}$$

Where:

DA_{event}	absorbed dose per event (mg/cm/event) $= Kp * C * t$	
	where	
	Kp (cm/h) is the coefficient of percutaneous permeation obtained in our study and by Ng *et al.*, 1992,	$0.27*10^{-5}$
	C is the concentration of DEHP applied to the skin (mg/L)	1.66
	t is the duration of exposure (h)	0.16
EV	event frequency (events/day)	1
ED	exposure duration (years)	1
EF	exposure frequency (days/year)	200
A	skin surface area (cm^2)	800
BW	body weight (kg)	70
AT	average time in days for non-carcinogens AT = ED	1

4.0 Results

When d_4-DEHP emulsified in cell culture media (RPMI) (aq) was applied to the skin, no d_4-DEHP nor non-deuterated DEHP (aq) were detected in the reservoir fluid (n=6). All the DEHP had metabolized and only mono-ester metabolite (d_4-MEHP) was detected. The mass accumulation curves for d_4-MEHP after dosing with neat d_4-DEHP and for DEHP (aq) are shown in Figs. 1 and 2, respectively. The τ, Kp, and J for d_4-DEHP are given in **Table 2**. Since no d_4-DEHP was detected in the reservoir fluid, we continued our experiments by applying DEHP (not deuterated) in the following experiments (n=18) as the results from the previous experiments showed no confounding from background DEHP contamination, and the non-deuterated chemical was a cheaper alternative. MEHP was not metabolized further in these experiments (no 5-oxo-MEHP or other metabolites were detected in the reservoir fluid). Overall, the lag-time quadrupled, the permeation coefficient reduced more than a hundred times, and the permeation

rate dropped 19-fold for neat compared to aqueous DEHP. The skin permeation resulting from applying neat DEHP was extremely low, while applying aqueous DEHP was considerable.

When MEHP emulsified in buffer (aq) was applied to the skin the lag-time was reduced 6-fold compared to applying neat DEHP and by 2h compared to emulsified DEHP (aq) (**Table 2**). Kp and J were increased considerably for emulsified MEHP compared to DEHP (**Table 2**). In the same experiment, the oxidized MEHP metabolite, 5-oxo-MEHP, was also detected. The mass accumulation curves for MEHP and 5-oxo-MEHP are shown in Figs. 3 and 4, respectively. The lag-time was the same for MEHP and 5-oxo-MEHP (6 h), while Kp was greatly reduced and J was almost 2000 times slower for the secondary metabolite.

The dermally absorbed dose (DAD) estimated for the maintenance task for (a) DEHP in aqueous solution was $2.85*10^{-2}$ mg/kg/day using our Kp ($15.1 * 10^{-5}$ cm/h) and $1.02*10^{-2}$ mg/kg/day using the Kp ($0.27 * 10^{-5}$ cm/h) obtained by Ng *et al.,* 1992 (Table 1); and (b) DEHP neat was $1.49*10^{-4}$ mg/kg/day (Kp $0.13 * 10^{-5}$ cm/h) (Table 2).

5.0 Discussion

Only the MEHP metabolite (100%) was detected in the receptor fluid when DEHP diluted in aqueous cell culture media (RPMI) was applied to human viable skin. DEHP application as neat or in aqueous solutions had an enormous impact on viable human skin permeation; neat DEHP hardly permeated skin and only did so after 30 hours, while the aqueous DEHP solution permeated skin after 8 hours as MEHP. MEHP diluted in aqueous cell culture media applied directly onto human viable skin permeated after only 6 hours. In addition, MEHP was oxidized further to 5-oxo-MEHP, although the permeation rate for the 5-oxo-MEHP metabolite was 2000 times slower than MEHP. The permeation rate (*J*) was 20 times slower when neat DEHP was applied to the skin compared to DEHP diluted in aqueous cell culture media. Our results show DEHP permeating human skin, contrary to some older human skin permeation studies which concluded that DEHP skin penetration is negligible (Barber et al., 1992). In the older studies, the total metabolites were less than 2.5% of applied dose compared to our 100%. This could potentially be due to a difference in esterase property between HPG and human skin. However, these older studies used non-viable skin, which might explain the differences observed. More

recent phthalate skin permeation studies (Beydon et al., 2010) have shown similar results to ours, namely that phthalates do permeate human skin but not as the parent compound but rather as metabolites.

In vitro skin permeation (Kp) previously reported for DEHP ranged from 0.011 x 10^{-5} (Barber et al., 1992) to 94 x 10^{-5} (Pelling et al., 1988) cm h^{-1} (**Table 1**), and ours fall in between (15 x 10^{-5} cm h^{-1} DEHP (aq)). This large variability in Kp might only partially be explained by the use of animal or human skin; but also the use of viable and non-viable skin, especially for chemicals metabolized in skin as we observed for DEHP. These earlier studies used heat separated skin, which after being immersed in 60ºC water would have lost considerably their enzyme activity, resulting in skin permeation due to diffusion only and not resembling what actually happens in human skin. In a study (Ng et al., 1992) comparing viable to non-viable dermatomed hairless guinea pig (HGP) skin, the authors found the viable skin to be metabolically active, producing twice the amount of metabolites compared to non-viable HGP skin.

Several factors govern the DEHP permeation rate through skin (permeability determinants), among those observed in our study were: DEHP solution applied (neat and aqueous solution), human skin (quality), and viability (assessed with TEWL), which determines the extent of metabolism. Other permeability determinants exist but were not explored in this study such as the choice of reservoir fluid, skin type (animal versus human, gender, age, type), storage (fresh and frozen), skin thickness, and SC separation methods (heat, NaBr, dermatomed). The vast number of permeability determinants complicates comparisons with other studies. Another large source of variability is between human skin samples, which might be underestimated in our study as only four donors were used for the experiments.

DEHP permeation rates are significantly influenced by the choice of receptor fluid. Receptor fluids should not restrict the permeation of the applied chemical (Howes et al., 1996); however, with very lipophilic substances such as DEHP the choice of receptor fluid is not clear. To overcome solubility restrictions, bovine serum albumin (BSA) is recommended added to the receptor fluid (OECD 2003); however, this addition should be questioned as 5% BSA have shown to inhibit skin metabolism, and therefore interfere with permeation (Haberland et al., 2006; Zhang et al., 2009). Using ethanol in the receptor fluid as in earlier experiments (Pelling et

al., 1998) will not allow for metabolism as the skin will not remain viable. Cell culture buffers (RPMI 1640 solution) may be good alternatives as they allow for skin metabolism, which has been shown in a recent study with di-butyl phthalate (DBP) (Beydon et al., 2010). For these reasons, we chose RPMI 1640 without BSA, which may explain the extensive DEHP metabolism observed in our experiments. Increased agreement between studies could be achieved if variability decreases, especially through promulgation of standardized guidelines for in vitro skin absorption studies (Ng et al., 1992) including how to handle possible dermal metabolism and to facilitate partitioning of metabolites into the receptor fluid, which may affect the biological activities of dermally applied compounds (Ng et al., 1992). Other variabilities are not inherent to the methods but rather due to human factors such as esterase activity necessary to hydrolyze DEHP to its metabolite MEHP, which differ between humans and genders (Zhang et al., 2009). Intra individual coefficient of variance was 21-109% for the mono ester metabolite of DBP in a human volunteer study (Janjua et al., 2008), and individual day to day variations were 17-78%.

Cutaneous esterases are located in the epidermis and in skin associated glands such as hair follicles (Muller et al., 2003), not in SC. Hair follicles are also in charge of skin homeostasis. Skin is capable of a variety of biotransformation processes including enzymatic process (however, much less compared to liver oxidation) (Bronaugh et al., 1994). Hydrolysis has also been observed in non viable skin (Bronaugh et al., 1994). Esterase activity might also differ between genders (Boehnlein et al., 1994). The importance of metabolism during percutaneous absorption depends on structure and biological activity of the penetrating compound and metabolite. For example in a very recent in vitro dermal study of [^{14}C]DBP applied to previously frozen human skin only MBP was found in the receptor fluid (Beydon et al., 2010). This was similar to our study where only MEHP was found in the receptor fluid after DEHP was applied to fresh human skin, suggesting that the esterase activity does not diminish after freezing. Beydon *et al.* (Beydon et al., 2010) also showed that inhibition of skin carboxylesterases decreased DBP skin permeation considerably, indicating that DBP itself cannot pass through skin without skin esterase hydrolysis. This might not be completely true as other studies (Scott et al., 1987) have shown that DBP and DEHP can penetrate the SC.

The SC is considered the rate limiting skin permeation step; however, in our study the rate was primarily determined by whether DEHP was applied neat or in aqueous solution. A chemical interpretation of this would be that DEHP, an extremely lipophilic substance, applied neat will reluctantly diffuse into the comparatively less lipophilic SC as seen with the long lag-time (>30 h), while aqueous DEHP have less affinity to water and prefer the lipophilic SC as seen with the much higher permeation rate. Once DEHP (aq) has permeated SC, it will immediately be hydrolyzed as shown with its similar permeation rates to the experiment with MEHP (aq) applied to the skin. In both cases, MEHP was measured in the reservoir fluid. This suggests that the rate limiting step is the availability of DEHP in the dermis (aqueous phase) for the aqueous DEHP was the diffusion through the SC rather than the metabolism of DEHP to MEHP by esterases. We could not evaluate the relative contribution of the two skin layers; epidermis and dermis, in our study because we did not use radio labelled DEHP and MEHP. Other researchers (Pelling et al., 1998) have found that in phosphate buffer during the first few hours after the lag phase, [^{14}C]DEHP absorption through the dermis (T_{lag} = 2.5h; Kp = 4.76 x 10^{-5} cm/h) was 3.7 times faster than through the epidermis (T_{lag} = 0.9h; Kp = 1.3 x 10^{-5} cm/h). In light of new research, we know that DEHP does not diffuse through the dermis but rather DEHP diffuses through SC and undergo metabolism in the dermis. It is currently very difficult to know which strata is the limiting factor. In a recent study (Beydon et al., 2010), permeation rates for MBP were 8–120 times faster than DBP for the same dose deposited, suggesting the DBP metabolism to be the rate limiting step of the percutaneous DBP absorption. Absent from their discussion was the application of DBP as neat and MBP as diluted in acetone, which can influence phthalate diffusion through SC as seen in our study and in an earlier study (Frasch et al., 2007) of the more volatile phthalate, diethyl phthalate (DEP). DEP skin permeation rates were almost double for DEP applied as a saturated aqueous solution compared to neat. From pharmaceutical research, it is known that the choice of vehicles can inadvertently cause changes in dermal absorption (Zhang et al., 2009). For example, alcohols (e.g. ethanol) used as solvents in topical dosing can interact metabolically by a transesterification reaction with the substrate of interest (e.g. inhibited the enzymatic conversion of beta-estradiol to estrone) (Oesch et al., 2007); and fatty ointments can impair enzymatic ester cleavage within the skin and reduce metabolism (Zhang et al., 2009). Moreover, chemical enhancers commonly used to increase drug delivery are alkyl esters. These

"prodrugs" increase skin permeation by being rapidly hydrolyzed in the epidermis creating "sink conditions" as observed when synthesized alkyl esters of morphine (more lipophilic than morphine) enhanced the delivery of morphine 2 to 5 times in aqueous solution while decreased delivery in oil (Zhang et al., 2009). Phthalates have two alkyl ester groups, making them excellent "prodrugs", as observed in our study where the two-alkyl ester DEHP increased the permeation of the mono-alkyl ester MEHP.

The total dermal dose DAD estimated for a work scenario changed drastically between the neat and aqueous DEHP (0.15 vs. 28.5 µg/kg bw/day, respectively) due to the differences in Kp. We compared our results to the study of Ng et al., (1992) because they also used dermatomed viable skin and a buffer as the receptor fluid as opposed to the two studies using human skin (Scott et al., 1987 used non-viable skin and 50% aqueous ethanol as the receptor fluid or Barber et al., 1992 who used non-viable heat separated human skin). The DAD calculated with our Kp was almost three times greater compared with the dose (10.2 µg/kg bw/day) calculated with an already published DEHP Kp (Ng et al., 1992). Both were above the daily intake calculated for the general population in Germany (Wittassek et al., 2007) (50^{th} percentile geometric mean 3.5 µg/kg bw/day), and US Environmental Protection Agency (EPA) reference dose (oral, 20 µg/kg bw/day), but below the European Food Safety Authority (EFSA) Tolerable Daily Intake (TDI) (50 µg/kg bw/day). Our values were similar to estimated daily intake (Hines et al., 2011) for workers manufacturing or using DEHP (Hines et al., 2008), suggesting that dermal uptake is not negligible for these workers.

Lipophilic chemicals will remain in the SC for some time before being absorbed into the general circulation. This phenomenon has in particular two regulatory implications when calculating the total absorbed dose (%). In an in vitro experiment, [^{14}C]DBP (7%, typical cosmetic exposure) was applied to skin and 27% of administrative dose was found in the receptor fluid while ~30% in the skin, of which 14% in the SC (Doan et al., 2010). Calculation of "total dose absorbed" depends on the intentions of DBP use; for an "industry setting" where OECD guidelines apply, DBP in the SC must be included (giving a total of 57% DBP absorption); while for "non-food" where European Union's Scientific Committee on Cosmetic Products and Non-Food Products Intended for consumers (SCCP, 2007) apply, DBP in SC must be excluded (giving a total of 43%

DBP absorption). (DEHP is not permitted for use in cosmetics in the European Union (Commission Directive 2004/93/EC)).

Under the EU Regulation (EC No 1907/2006) concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) exposure assessments including the dermal route have to be calculated. The validity of the exposure assessment depend on existing dermal absoption studies. In general, these studies are few and inconsistent (also true for phthalates, see Table 1), and often based on animal data, which cannot be readily applied for humans as human skin is less permeable (IOM, 2004). This is not the case for DEHP where the Kp obtained using viable dermatomed Guinnea pigs were 56-fold lower than what we obtained in this study using viable dermatomed human skin, leading to an underestimate of the dermally absorbed dose for aqueous DEHP. The difference is in the extensive metabolism observed in human fresh skin.

Although a regulatory binding biological monitoring limit value has not been set for phthalates as of yet, the use of the urinary phthalate metabolites have been used extensively to assess total phthalate exposures. The validity of biological monitoring must be questioned as the timing of the sample which relates to the time between exposure and time for blood or urine sampling determines the collection time. Failure to consider the lag time between dermal exposure and appearance of phthalate or metabolites in the general circulation may cause false conclusions about exposure (Nielsen and Nielsen, 2000). The optimal collection time in our hypothetical maintenance scenario would be after dermal permeation (T_{lag} of 8h) and clearance from blood (estimated by the parenteral route (Pollack et al., 1985; Sjoberg et al., 1985) (half-life of 10 h), meaning urinary collections should start 18 h after the task started. Several occupational exposure studies (Gaudin et al., 2011; Gaudin et al., 2008; Koch et al., 2012) have found pre-shift urine samples to contain elevated phthalate metabolite concentrations, which support the hypothesis postulated by Koch *et al.*, (Koch et al., 2012): “the maximum elimination was not achieved directly after the shift”, and this would be due to the dermal exposures associated with longer clearance times.

Exposure to DEHP remains important with respect to human exposures as it can impair fertility to humans or cause development toxicity in humans (classified in the EU as category 2 substances to reproduction). Absorption of DEHP through the dermal route is significant, and not as currently regarded: negligible or low. In addition, skin is an endocrine organ containing estrogen receptors (ER_α and ER_β) (Thornton, 2002). DEHP and MEHP can bind to estrogen receptors. Both DEHP and MEHP have shown low antiandrogenicity effect in an *in vitro* study (Christen et al 2010), thus the endocrine activity would not be effectively reduced in the first DEHP metabolism step. What endocrine effects this might exert is currently unknown and needs further scrutiny.

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