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Human skin absorption of three phthalates

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ABSTRACT

Population studies reveal widespread exposure to phthalates. Understanding their absorption, distribution, metabolism, and excretion is vital to reduce exposure. However, data on skin absorption remain limited. We thus aim to characterize the skin permeation of three phthalates in a mixture, neat or in emulsion; di(2-ethylhexyl) phthalate (d4-DEHP), dibutyl phthalate (d4-DBP), and diethyl phthalate (d4-DEP), by comparing in vitro human skin (800 μm) permeation (24 hours) results using flow-through diffusion cells with urine results obtained from volunteers exposed to the same mixture applied to a forearm (40 cm^2). Metabolites were analyzed in receptor fluids and urine. Phthalates crossed the skin barrier and metabolized into monoesters before elimination. Increased permeation was observed for phthalates in emulsion compared to neat substances, with polyethylene glycol (PEG) in the receptor fluid enhancing emulsion permeation, but not affecting neat substances. In vitro results mirrored in vivo findings: DEP showed rapid permeation (J: ~2 ug/cm^2/h) and urinary excretion peaking at six hours post-application, whereas DBP exhibited slower kinetics (J: ~0.1 ug/cm^2/h), with a urinary peak at 15–17 hours post-application. DEHP had minimal permeation (J: ~0.0002 ug/cm^2/h) with no observable urinary peak. These findings underscore the importance of comprehending phthalate skin absorption for effective exposure mitigation strategies.

1. Introduction

Research has shown that exposures to phthalates is associated with various disorders, but most significantly with reproductive disorders (Hlisníková et al., 2020). They are used in many different professional and consumer products to impart elasticity, flexibility, and durability, as plasticizers, but also for their solvent properties (Hines et al., 2009). Phthalates are not chemically bound to the polymer and can migrate to the environment.

Phthalate exposures are ubiquitous in the environment and have been detected in indoor air (Becker et al., 2002), food and other materials (Kumar and Maitra, 2016) as well as cosmetics (Olkowska and Gržinić 2022). Biomonitoring studies focusing on the general population in the US (Silva et al., 2004) and Germany (Koch et al., 2017) have highlighted that phthalate metabolites are consistently present in urine, indicating widespread exposures.

Phthalates enter the body mainly by ingesting food and drinks for the general population but also by inhaling phthalates found in the air and by skin when in contact with phthalate containing products (Zhao et al., 2022) (Hlisníková et al. 2020; Benjamin et al. 2017). Toxicokinetic research has focused on ingestion and to a lesser degree inhalation and dermal routes of exposure. This is because the relative importance of skin exposure compared to ingestion is lower (Giovanoulis et al., 2018). The significance of dermal uptake is usually governed by molecular weight and water solubility according to Fick's law of diffusion. Phthalate skin absorption increases from high molecular weight

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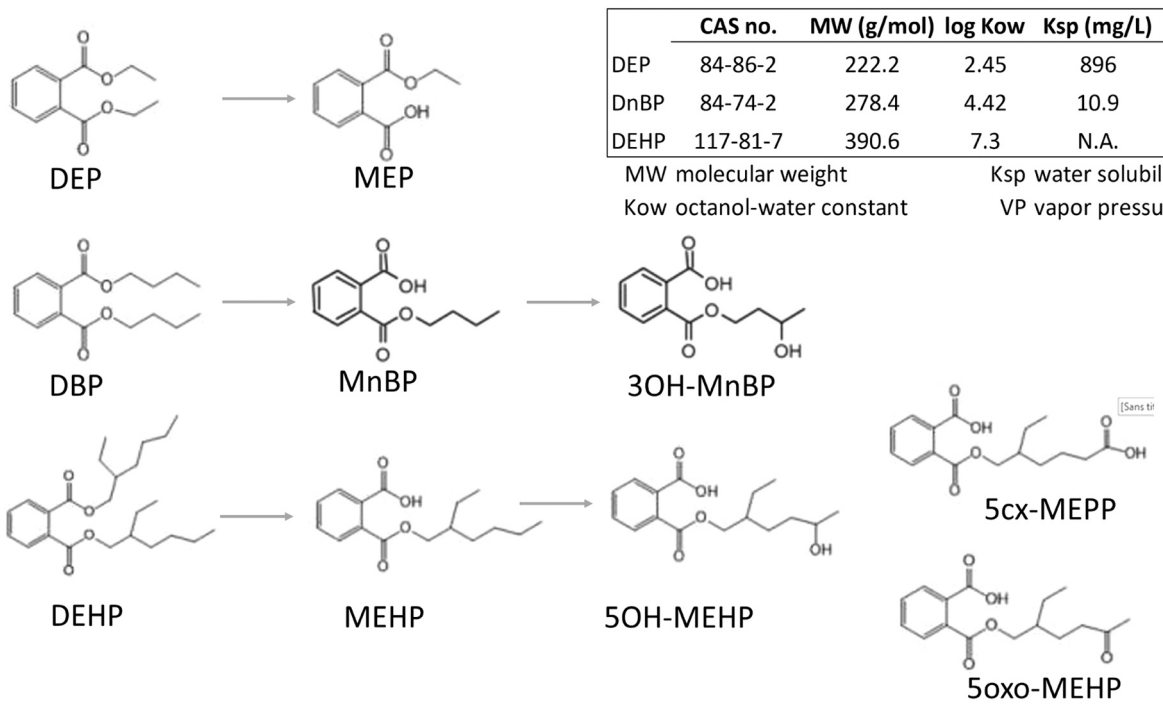
phthalates to low molecular weight phthalates (Giovanoulis et al., 2020). Highly lipophilic substances, such as phthalates, are assumed to have low skin absorption, but the skin's ability to metabolize phthalates may alter their uptake rates (Hopf et al., 2014). Skin exposure assessments that disregard this metabolic activation might therefore underestimate the body burden from this route of entry in health risk assessments (Hu et al., 2022). This is especially important for phthalates considering that the monoester metabolites have direct access to the systemic circulation and thus, the target organs (Hauser and Calafat, 2005; Sugino et al., 2017).

Phthalates are also metabolized once absorbed into the body. The first step is hydrolysis by esterases (in lungs, skin, and the digestive system), which yields the primary metabolite, a monoester phthalate (Frederiksen et al., 2007). Then decarboxylations can lead to several oxidation products depending on the alkyl chain length oxidations (Koch et al., 2005). Both, the simple and the oxidized monoesters, are eliminated fully or partially (depending on their hydrophilicity) in their conjugated form produced by uridine 5'-diphosphoglucuronyl transferase.

Here, we focus on three common phthalates with different physical and chemical properties: diethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), and diethyl phthalate (DEP) (Domínguez-Romero and Scheringer, 2019) (Fig. 1). DEHP was the most used phthalate plasticizer in polyvinyl chloride (PVC) products and DBP in resins and polymers (Szewczyńska et al., 2020). However, they were included in the Regulation (EC) 1907/2006 on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH, annex XIV) as substances of very high concern (SVHCs), and their production and import were forbidden in July 2021 in Switzerland. Both phthalates are classified as toxic to the reproductive system (Europe (EU): category 2 and Globally Harmonized System (GHS: 1B). DEP is a low molecular weight phthalate mainly used for its solvent properties in personal care products, industrial materials, and medical products (Ellington and Floyd, 1996; Wang and Qian, 2021).

The metabolic pathways of DEHP, DBP, and DEP are given in Fig. 1. DEHP is first hydrolyzed by the ubiquitous esterases to the corresponding monoester, MEHP (Koch et al., 2005). MEHP is oxidized to several secondary oxidation metabolites: mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP) and mono-[2 (carboxymethyl) hexyl] phthalate (2cx-MEPP) (Koch et al., 2005). The metabolism of DBP produces the corresponding mono ester MBP and one oxidized secondary metabolite; mono-(3-hydroxybutyl) phthalate (3OH-MBP) (Koch et al., 2012). The oxidized metabolites are less dominant because its alkyl chain is shorter. Two DBP isomers exist: normal- or DnBP and iso- or DiBP. Fig. 1 depicts the DnBP, which usually more abundant. DEP is only metabolized to its monoester (MEP) with no further oxidation (Jang et al., 2020) (Fig. 1).

Once phthalates have been absorbed into the stratum corneum (outer layer of the skin), they diffuse through and gain access to viable epidermis, where the esterases can metabolize the parent compound to monoesters (Bätz et al., 2013). The skin permeation rates from human *in vitro* studies are highly variable and reported to be: 0.1–2.2 μg/cm^2/h for DEHP (Barber et al., 1992; Hopf et al., 2014; Scott et al., 1987), 0.07–0.59 μg/cm^2/h for DBP (Beydon et al., 2010; Scott et al., 1987), and 1.27–21 μg/cm^2/h for DEP (Barbero and Frasch, 2016; Mint et al., 1994; Scott et al., 1987). The *in vitro* quantitative results were highly variable between studies, and this is probably due to differences in the anatomical origin and thickness of skin used as well as experimental conditions (exposure duration, concentration, vehicle, and experimental apparatus). Skin absorption in human volunteers has been demonstrated for DEHP dissolved in ethanol ([^{14}C]DEHP) (Wester et al., 1998) as well as DEP and DBP (2 % (w/w) in a cream formulation over a week (Janjua et al., 2007). None of the studies have, however, shown how well the extrapolation conducted in risk assessment from the results obtained with *in vitro* flow-through skin permeation experiments agrees with *in vivo* human skin exposure assessed by human biomonitoring (quantification of metabolites in blood and urine) for these highly lipophilic



	CAS no.	MW (g/mol)	log Kow	Ksp (mg/L)	VP (Pa)
DEP	84-86-2	222.2	2.45	896	0.08
DnBP	84-74-2	278.4	4.42	10.9	0.003
DEHP	117-81-7	390.6	7.3	N.A.	0.0005

MW molecular weight Ksp water solubility constant
Kow octanol-water constant VP vapor pressure at 25C

Fig. 1. DEP, DBP, and DEHP are metabolized by esterases to their corresponding monoesters. Other metabolites exist but only the metabolites analyzed in this study are shown here. Mono-2-ethylhexyl phthalate (MEHP) undergoes further metabolism to the oxidized side-chain metabolites: 5OH-MEHP, 5cx-MEPP, and 5oxo-MEHP; and mono-n-butyl phthalate (MnBP) to 3OH-MnBP. (Physical chemical constants from The Handbook of Environmental Chemistry Vol. 3, Part Q (2003): 57–84 DOI 10.1007/b11463).

substances that are metabolized in skin.

Our goal was to address these limitations associated with extrapolation from *in vitro* to human exposures. We performed *in vitro* skin permeation experiments with *ex vivo* human skin applying a mixture of deuterated DEHP, DBP, and DEP (objective 1) and applied the same phthalate mixture to one forearm of human participants in a toxicokinetic study (objective 2). Finally, we explored the agreement between the *in vitro* skin permeation results to human skin absorption studies (objective 3). For objective 1, we applied a controlled dose of the deuterated phthalate mixture to previously frozen human skin *in vitro* to characterize skin permeation using two different polyethylene glycol (PEG) concentrations (0.006 and 6 % PEG) in the receptor liquid to understand possible absorption barriers. For objective 2, we used the same phthalate mixture used in the *in vitro ex vivo* human skin experiments, applied it to a delimited skin area on one forearm of five participants, and quantified the extent of absorption, distribution, metabolism, and excretion of these phthalates over 24 hours. In objective 3, we compared our *in vitro ex vivo* human skin results with the human participant results.

2. Methods

2.1. Chemicals

The following chemicals were purchased d4-DEHP (CAS no. 93951–87–2; density 0.98 g/mL), d4-DBP (CAS no. 93952–11–5; density 1.046 g/mL) and d4-DEP (CAS no. 93952–12–6; density 1.12 g/mL) (Sigma Aldrich, Switzerland). For the *in vitro* skin permeation experiments, the following chemicals were purchased: methanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), sodium chloride (purissim. p.a. $\geq$ 99.5 %, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), and polyethylene glycol (PEG) (Brij® Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Purified water was produced with a Milli-Q® Advantage ultra-pure water system (Millipore, Milford, MA, USA).

2.2. In vitro skin permeation

2.2.1. Phthalate mixture preparation

Two mixtures were prepared; each with the three phthalates: a mixture of neat phthalates and a mixture with the same phthalates emulsified in water with 1 % methanol (v/v). Both mixtures were prepared with deuterated phthalate standards using precision glass syringes and a precision balance. To prepare the mixture of neat phthalates, the phthalates were weighed in the same vial and then mixed using a vortex mixer. To prepare the mixture of phthalates emulsified in water, the phthalates were weighed in the same vial, then dissolved in methanol, and finally, water was added to achieve the desired concentrations. The mixture compositions reported in percent weight were as follows: Neat A: 26.4 %, 33.9 %, and 39.7 % for d4-DEHP, d4-DBP, and d4-DEP, respectively; Neat B1: 45.6 % and 54.4 % for d4-DEHP and d4-DBP, respectively; Neat B2 was neat d4-DEP. Emulsion A: 169 μg/mL, 174 μg/mL, and 153 μg/mL for d4-DEHP, d4-DBP, and d4-DEP, respectively; Emulsion B1: 167 μg/mL for both d4-DBP and d4-DEHP; Emulsion B2: 2360 μg/mL for d4-DEP. After applying Neat A and Emulsion A mixtures, which contained DEHP, DBP, and DEP, we decided for analytical reasons to apply separately DEP from DEHP and DBP. Therefore, we prepared Neat mixture B1 and Emulsion B1 containing DBP and DEHP, and Neat mixture B2 and Emulsion B2 containing DEP only.

2.2.2. Human skin

Skin from patients undergoing abdominoplasty at the Department of Plastic and Reconstructive Surgery at the Cantonal University Hospital (Centre Hospitalier Universitaire Vaudois) (CHUV) in Lausanne, Switzerland, was collected immediately after surgery, as described by the Department of Musculoskeletal Medicine (Wei et al. 2017) biobank. The surgeon obtained patient consent for the use of the removed skin in our experiments according to the human ethics approval by the Swiss ethics CER-VD ("commission cantonal d'éthique de la recherche sur l'être humain") (ethical protocol 264/12). No personal information regarding the patients (gender, age, ethnicity, BMI) was retained. This skin was dermatomed (Acculan®II, B. Braun/Aesculap, Sempach, Switzerland) to a thickness of 800 μm, wrapped in aluminum foil, and frozen at −20°C until experiment day.

2.2.3. Experimental design

On experiment day, the skin was defrosted in the aluminum foil and cut into circular discs. We used skin from four different donors and ran the experiments in triplicates for each donor skin. The skin discs were mounted onto flow-through diffusion cells (receptor volume 8.5 mL; 11.28 mm diameter opening; PermeGear bought from SES Analytical System, Bechenheim, Germany). Diffusion cells were composed of a donor chamber and a receptor chamber separated with a dermatomed human skin disc. The exposed skin area was 1 cm^2. The receptor chamber was filled with saline (0.9 % NaCl in water) and polyethylene glycol (PEG; either 0.6 % or 0.006 % w/w). The diffusion cells were connected to a peristaltic pump (Ismatec IPC-N, IDEX Health & Science GmbH, Wertheim-Mondfeld, Germany) set at 40 μl/min, and the replaced receptor fluid was collected in a fraction collector (FC 204, Gilson Inc., Middleton, WI, USA). The flow-through system operated at 32°C. The skin discs were then stabilized for approximately 30 min, as 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 experiment started when the diffusion cells were dosed with a mixture of either neat phthalates (10 μl/cm^2) or phthalates emulsified in water with 1 % MeOH (847 μl/cm^2) (Hopf et al., 2014). Table 1 summarizes the applied skin dose (mg/cm^2) for neat and emulsified phthalate mixtures for the *in vitro* experiments and participants' forearms (explained later in the description of the participant study, see Section 2.3). The automated fraction collector was set for three-hour intervals lasting 24 hours. The control samples were run without anything applied to the skin. After 24 hours, the collected receptor fluid samples were frozen at −20°C.

In the Neat B2 and Emulsion B2 mixtures, the amount of DEP was increased compared to the Neat A and Emulsion A mixtures to try to achieve favorable conditions for reaching steady state (see Section 3.1).

2.2.4. Semi-mass balance experiments

Mass balance is calculated for *in vitro* skin permeation studies to quantify the tested substance in: (1) the receptor fluid that have crossed the skin barrier and are systemically available to circulate to other organs; (2) the skin following a wash, which have the potential for systemic availability; and (3) the diffusion cell wash. The total mass balance for substances that are not radiolabeled has an acceptable range 80–120 % (OECD GD28, par. 77; GN156, par. 95). We quantified phthalate metabolites, not the parent compounds in this study, and therefore called our approach a semi-mass balance. The semi-mass balance protocol was as follows:

- the remaining dosed liquid on top of the skin was removed (donor chamber);
- the skin surface was cleaned three times with MilliQ water (skin washing);
- the pipette tip used for applying neat phthalate mixture (pipette tip) and for cleaning the skin surface (tip rinsing) was rinsed twice with MeOH;
- the donor chamber was rinsed three times with MeOH (donor chamber washing);
- the remaining receptor fluid in the receptor chamber was collected (receptor chamber);

Table 1
Doses for each deuterated phthalate (DEHP, DBP, and DEP) standard applied per square centimeter of human skin (mg/cm^2) in the *in vitro* experiments as either neat (two different mixtures A and B) or as an emulsion: Mixture of the phthalates emulsified with 1 % MeOH in water (tree different mixtures: A, B1 and B2). Only the neat mixture was used for the human participant study.

		d_4-**DEHP** (mg/cm^2)	d_4-**DBP** (mg/cm^2)	d_4-**DEP** (mg/cm^2)	d_4-**DEHP** (μmol/cm^2)	d_4-**DBP** (μmol/cm^2)	d_4-**DEP** (μmol/cm^2)
In vitro							
	Neat A	2.8	3.5	4.1	7.0	12.6	18.3
	Neat B1	4.6	5.5	0	11.7	19.6	0
	Neat B2	0	0	9.0	0	0	39.6
	Emulsion A	0.14	0.15	0.13	0.4	0.5	0.6
	Emulsion B1	0.14	0.14	0	0.4	0.5	0
	Emulsion B2	0	0	2.0	0	0	8.8
Participants							
	Neat	4.0	4.0	2.0	10.1	14.2	8.8

Section 2.2.1 describes the different mixtures.

- the skin disc was sonicated three times in MeOH for 5 minutes (skin).

The semi-mass balance was expressed in μmol equivalents of the parent compound. The mean values were calculated over all cells and were reported as percent of the administered dose of the parent compounds. All samples were frozen at −20°C and shipped for metabolite analyses to the Institute for Prevention and Occupation Medicine (IPA) in Germany under dry-ice and arrived frozen.

2.2.5. Skin permeation parameters

The cumulative amount per unit skin area of the phthalate metabolites in the receptor fluid was plotted as a function of time (hours) generating a permeability curve. Skin permeation parameters were determined from the permeability curve: the permeability rate J (μg/cm^2/h), the lag time Tlag (h), and the permeability coefficient Kp (cm/h). J defines how fast the substance crosses the skin and was calculated as the slope of the steepest part of the permeability curve. The averages and the standard deviations of J for all diffusion cells were calculated for each substance. The Tlag defines how long the substance needs before steady state is reached and it can be estimated with the intercept of the steady state portion of the permeability curves (the steepest linear part of the permeation curve) with the time axis. The Kp was calculated as the ratio between J at the steady state and concentration of the chemical applied on the skin, or the density of the chemical when phthalates were applied neat. Steady state is mandatory to calculate the two kinetic parameters: Kp and Tlag. 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 duration of sampling period (24 h).

2.3. Human participant study

2.3.1. Participant recruitment

We recruited participants in Lausanne (VD, Switzerland) with electronic flyers and distributing flyers at different buildings in Lausanne. Inclusion criteria were 18–65 years old, non-smoking, body mass index (BMI) between 18 and 25, not under a medical regime, and not pregnant. Five participants were recruited, and they signed the written informed consent before the exposure session. Participants received a compensation for completed study participation. This study was approved on 14 October 2020 by the Swiss Ethics in Human Research, Canton de Vaud (CER-VD) ("commission cantonal d'éthique de la recherche sur l'être humain") (ethical protocol 2020–01095).

2.3.2. Study design

We applied 400 μL of a deuterated mixture of neat d4-DEHP, d4-DBP, and d4-DEP (see Table 1) to a skin area of 40 cm^2 of one forearm on each participant. The treated skin area was left uncovered and unwashed for a period of 6 hours. The doses were adjusted to ensure a higher dose of DEHP and DBP and a lower dose of DEP, knowing that the first two would permeate the skin less compared to DEP, and thus wanting to create conditions where all three phthalates could be quantified in the participant's urine. The choice of a 40 cm^2 area on one forearm was the largest skin area that allowed the mixture to not drip off the forearm, while maintaining the same volume of phthalate mixture per skin surface as in the *in vitro* experiments (10 μL/cm^2). Participants were exposed to one forearm only, as applying to both forearms and not being able to use either arm during six hours would deter participation. The 6-hour exposure period for participants was determined considering participant comfort and practicality in a controlled setting. Participants were under rest condition (no physical activity) during the whole exposure in a controlled setting (exposure chamber constructed of metal). Participants were asked to donate a urine sample before we applied the mixture to the forearm, every two hours during exposure, and *ad lib* for the following 24 hours. Participants were asked to measure the urine volume, record it, and bring the urine samples back to the laboratory the next day. Urine sampling cups used to collect urine samples were without phthalates and any other potential (background) contamination was avoided by dosing deuterated phthalates. Urine samples were stored at −20°C and again shipped to IPA for analysis, as described for the *in vitro* samples.

2.3.3. Kinetic parameters

Two kinetic values were calculated: percentage of applied dose and apparent urinary elimination half-lives. We summed the total amount of metabolites recovered in urine collected up to 30 h post-application (μmol equivalent of parent compound) and calculated the arithmetic means and standard deviations for percentage of applied dose. We calculated the half-lives using the urinary elimination curves to find the time when the concentration was half of Cmax (the maximum urinary concentration of the metabolites).

2.4. Chemical analysis of metabolites

The chemical analytical method for quantification of phthalate metabolites has been described previously (Koch et al., 2017). Briefly, urinary conjugates are hydrolyzed (β-glucuronidase) and chromatographic separation was performed by an automated on-line two dimensional HPLC configuration with tandem-MS detection (Koch et al., 2017). As dosing was performed with the d4-labelled parent phthalates and target analytes were the d4-labelled metabolites (used in the original analytical method as internal standards), their quantification needed to be performed using either 13C4-labelled internal standards (13C4-MEHP; 13C4–5OH-MEHP; 13C4-MnBP) or by standard addition at three concentration levels. Limits of quantification (LOQ) were between 0.06 μg/L for d4-MEHP and 0.2 μg/L and 1.0 μg/L for all the other metabolites. No measurable background contamination with

d4-derived metabolites was observed in blank samples. The IPA participated in external quality assessment schemes for phthalates and obtained valid certificates for all biomarkers analyzed in the current study at HBM4EU (Esteban Lopez et al., 2021; Mol et al. 2022) and G-EQUAS (Göen et al., 2012).

3. Results

3.1. In vitro skin permeation of phthalates

Table 2 shows the values obtained for the *in vitro* skin permeation parameters (J, Tlag and Kp) for all phthalates (DEHP, DBP and DEP) after application of neat and emulsified phthalate mixtures with receptor fluid containing two concentrations of PEG (0.006 % and 6 %). Only the monoesters, not the oxidation metabolites, were found in the receptor fluid, in all experiments. MEHP reached steady state only when applied neat and with 0.006 %PEG in the receptor fluid, otherwise it was below the limit of detection.

Fig. 2 shows the skin permeation curves for MEHP, MBP, and MEP. Permeation rates were calculated from the steepest linear of these curves, and were for MEHP 10–24 hours (emulsified), MBP 10–20 hours, and MEP 6–11 hours.

DEP permeated the skin readily as MEP and to the extent that it was depleted (as neat with 0.006 % PEG and emulsion with 6 % PEG). This effect was observed in the cumulative dose plots as a plateau of the curve. We reached steady state in the experiment with neat DEP and 6 % PEG in the receptor fluid after doubling the dose, while emulsified DEP did not. DBP reached steady state in all experiments except when applied as an emulsion with 6 % PEG in the receptor fluid.

The mean percentages of the administered doses that were metabolized into monoesters and detected in the different compartments are shown in Table 3. Pipette tips were rinsed after applying the neat mixture because we observed droplets adhering on the inside walls of the pipette tips. Tip rinsing after skin washing was not included initially but was included in the second set of experiments (6 % PEG). Most of the tip rinsing samples were close to the LOQ. No donor chamber samples were collected for neat mixture experiments because we did not observe any liquid on top of the skin after 24 hours.

Table 2
In vitro skin permeation parameters for DEHP, DBP, and DEP estimated from their permeability curves. A mixture of DEHP, DBP, and DEP was applied on the skin in vitro for experiments with 0.006 % PEG in the receptor fluid. DEP was applied on the skin in vitro alone while DEHP and DBP was applied as a mixture on the skin in vitro for experiments with 6 % PEG in receptor fluid.

Application	n	PEG (%)	Metabolites	Kp ± SD (10^{-6} cm/h)	J ± SD (ug/cm^2/h)	T lag ± SD (h)
Neat A	9	0.006	MEHP	0.0002 ± 0.0001	0.0002 ± 0.0001	9.4 ± 3.9
			MnBP	0.10 ± 0.03	0.11 ± 0.04	4.6 ± 1.4
			MEP*	n.a.	1.22 ± 0.59	n.a.
Neat B1	3	6	MEHP	n.d.	n.d.	n.d.
			MnBP	0.08 ± 0.03	0.08 ± 0.03	6.9 ± 1.1
Neat B2	3	6	MEP	1.71 ± 0.80	1.91 ± 0.89	3.9 ± 0.4
Emulsion A	9	0.006	MEHP	n.d.	n.d.	n.d.
			MnBP	2280 ± 810	0.38 ± 0.13	7.5 ± 1.9
			MEP	19144 ± 7032	3.18 ± 1.17	3.5 ± 1.2
Emulsion B1	3	6	MEHP	n.d.	n.d.	n.d.
			MnBP*	n.a	0.24 ± 0.06	n.a.
Emulsion B2	3	6	MEP*	n.a.	7.02 ± 2.61	n.a.

n.d.: chemicals not detected (under the LOQ) in the samples.
n.a.: not available
*: did not reach the steady state.

3.2. In vivo skin permeation

3.2.1. Urinary elimination

Five participants participated in our study and had a mean body mass index of 21.9 (±1.8) and mean age of 27.5 (±10) years. We applied neat phthalate mixture to participants' forearms because the emulsified mixture simply ran off while the phthalate mixture, which is more viscous would remain on the skin. We detected the full range of monoester and oxidized metabolites in urine after the *in vivo* skin permeation. The molar sum of all urinary metabolites quantified for each phthalate up to 30 hours post-application was 3 % (± 2 %) of the applied dose for DEP (only MEP), 1 % (± 0.5 %) for DBP (MBP + 3OH-MBP), and 0.01 % (± 0.003 %) for DEHP (MEHP + 5OH-MEHP + 5oxo-MEHP + 5cx-MEPP). Mean urinary concentrations (creatinine corrected) of the respective individual phthalate metabolites after skin exposure to DEP, DBP, and DEHP are shown in Fig. 3 as cumulative concentrations over time (left column) and time dependent elimination concentrations (right column). We aligned the urine void times to the nearest hour to produce these graphs.

For DEP, urinary elimination of MEP started after two hours post-application and continued to rise until a maximum cumulative concentration was reached for all participants around six hours post-application (Fig. 3A). No MEP (d4-MEP) was detected in the participants' urine prior to exposure. Most of the MEP had eliminated within 24 hours post-application. The MEP half-life was about eight hours (Fig. 3D).

For DBP, urinary elimination kinetics for MBP and 3OH-MBP were similar. The predominant urinary metabolite was the monoester MBP, at concentrations more than an order of magnitude higher than 3OH-MBP (Figs. 3B and 3E). The sum of the two are shown in Fig. 3B. We detected urinary DBP metabolites after two hours post-application. Again, none of the deuterium labelled DBP metabolites were detected in the participants' urine prior to exposure. The urinary MBP and 3OH-MBP concentrations continued to rise until maximum concentration was reached for all participants between 13 and 23 hours post-application of the phthalate mixture to the skin. The urinary elimination graphs show no obvious peak. Therefore, the half-life was estimated to be 2–9 hours. Although, most of MBP and 3OH-MBP had eliminated within 30 hours post-application their presence was still measurable.

For DEHP, urinary elimination kinetics for MEHP, 5OH-MEHP, 5oxo-MEHP, and 5cx-MEHP were rather similar, and concentrations were very low (Figs. 3C and 3F), compared to the metabolites of the other two phthalates. The oxidized metabolites were excreted at higher concentrations than the monoester MEHP. The intra-individual variability was considerable on the day of the exposure. We detected all urinary DEHP metabolites four hours post-application in four of the five participants. One participant started eliminating all DEHP urinary metabolites two hours post-application. No deuterium labelled urinary DEHP metabolites were detected in the participants' urine prior to exposure. All participants had a slow and continued rise in urinary DEHP metabolite concentrations even after 24 hours post-application. Maximum concentrations were not reached after 30 hours post-application. Consequently, we cannot report a half-life for these metabolites.

4. Discussion

The relative importance of the skin exposure route for very lipophilic substances such as phthalates is less compared to ingestion (Giovanoulis et al., 2018); however, the skin route of entry adds to the overall body burden and should be considered in human health risk assessments. Dermal uptake is very modest for high molecular weight phthalates (Giovanoulis et al., 2020), as our DEHP results confirm, but can be

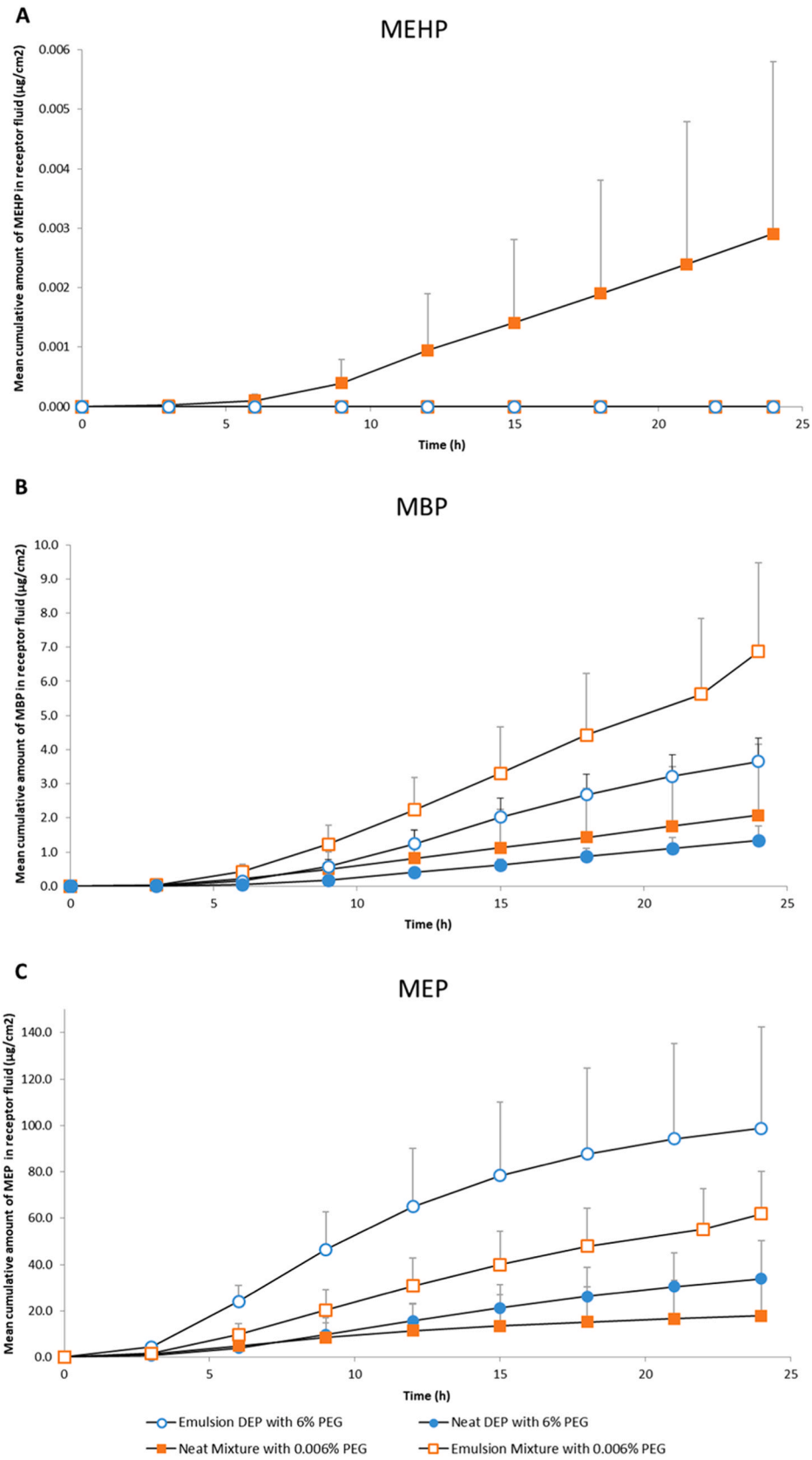


Fig. 2. In vitro metabolite permeability curves with mean cumulative metabolite amounts in the receptor fluids (ug/cm2) (y-axis) were plotted over time (h) (x-axis) for (A) DEHP quantified as MEHP, (B) DBP quantified as MBP, and (C) DEP quantified as MEP. None of the secondary metabolites were detected in the receptor liquid. Parent compounds were not analyzed. Applied substance doses are given in Table 1 and the chemical structures in Fig. 1. The means and standard deviations (one sided shown to better visualize the curves) (n= 3–9, Table 2) of the cumulative amount of primary phthalate metabolites in the receptor fluid are plotted over time.

Table 3
Semi-mass balance expressed as percentage of the applied dose (in moles) to the in vitro skin samples.

PHTHALATES												
	NEAT						EMULSION					
	6 % PEG			0.006 % PEG			6 % PEG			0.006 % PEG		
	D4-MEHP	D4-MBP	D4-MEP	D4-MEHP	D4-MBP	D4-MEP	D4-MEHP	D4-MBP	D4-MEP	D4-MEHP	D4-MBP	D4-MEP
Pipette tip neat	0.0005	0.0004	0.0012	0.0002	0.0002	0.0104	NA	NA	NA	NA	NA	NA
Tip rinsing	0.0008	0.0013	0.0039	NA	NA	NA	0.0019	0.0757	NA	NA	NA	NA
Donor washing	0.0012	0.0009	0.0119	0.0009	0.0009	0.0235	0.0004	0.0099	0.0910	0.0014	0.1079	0.8059
Skin	0.0016	0.0092	0.1458	0.0002	0.0002	0.0098	0.0012	1.0578	0.6094	0.0010	1.0409	0.6835
Skin washing	0.0040	0.0026	0.2064	0.0042	0.0042	0.1654	0.0272	3.9010	0.0158	0.0023	0.1183	0.1457
Donor chamber	NA	NA	NA	NA	NA	NA	0.0823	33.4627	0.4316	0.0210	0.4837	1.7281
Total receptor chamber	<LOQ	0.3246	5.5415	0.0013	0.6368	4.2006	<LOQ	38.9116	75.4411	<LOQ	46.5614	49.9050
TOTAL	0.0083	0.3391	5.9108	0.0068	0.6510	4.4097	0.1129	77.4187	76.5888	0.0256	48.3122	53.2682

NA: not available; LOQ: limit of quantification, values are based on the monoester concentrations (not the parent phthalate concentrations).

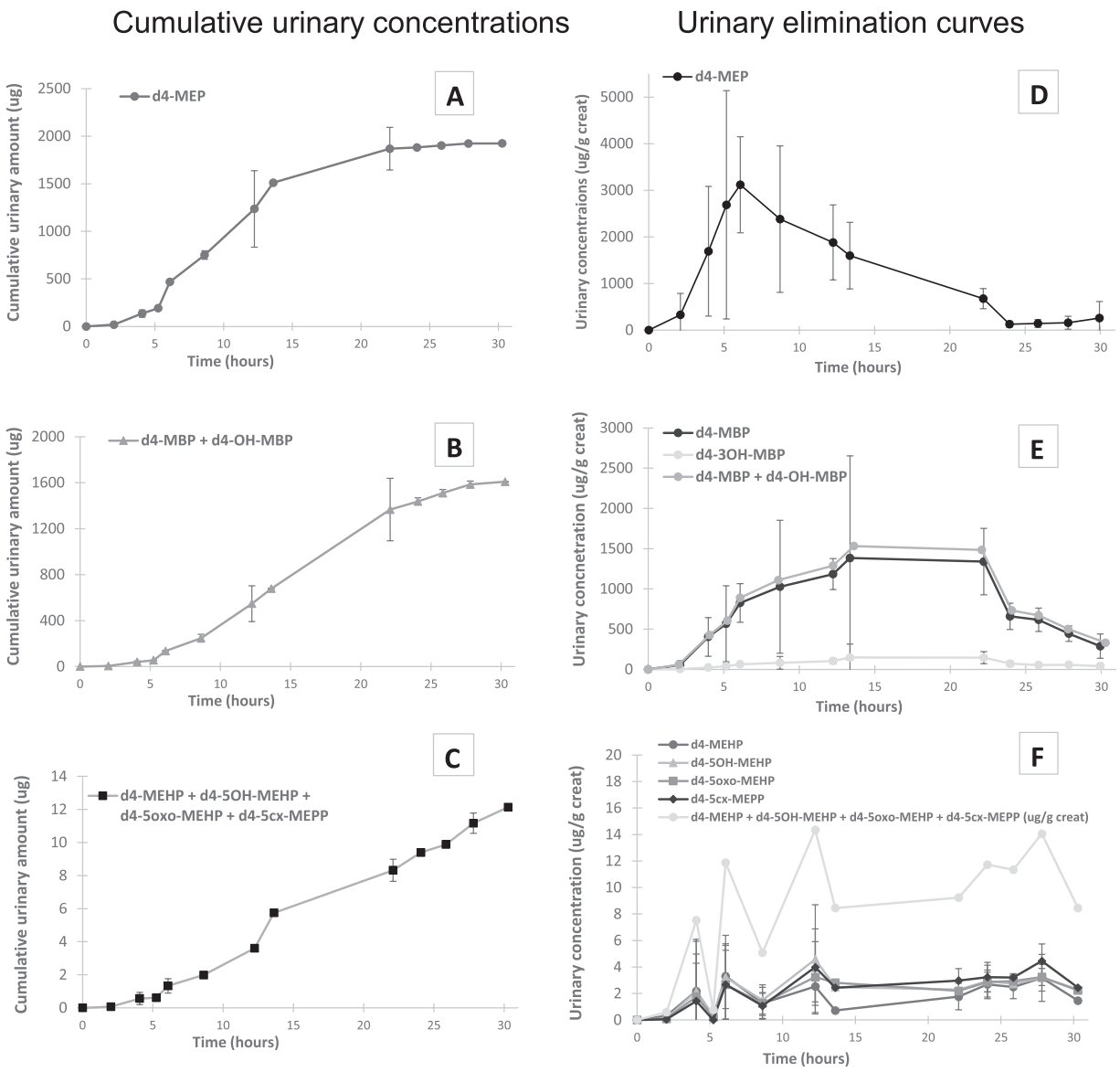


Fig. 3. The left column depicts the cumulative urinary concentrations over time for MEP (A), the sum of two DBP metabolites (B), sum of four DEHP metabolites (C), and the right column time dependent elimination concentrations over time for MEP (D), two DBP metabolites (E), four DEHP metabolites (F). Time 0 indicate the time of application of phthalate mixture to one forearm of the participants. Note that the y-axis concentrations differ by several orders of magnitude from DEP to DEHP.

significant for low molecular weight phthalates (Wormuth et al., 2006), which is in line with what we observed for DEP. In our study, we were able to avoid both any influence of the known omnipresent background exposures to phthalates in participants and any detrimental interference by analytical contamination by using deuterium labelled parent phthalates for our skin permeation study. Analytical blanks were always below the LOQ, and the pre-dose human urine samples did not contain any measurable metabolite levels of the respective d4-phthalates. The parent compounds were not measured in our study because we did not have reliable methods to quantify these. Below, we discuss the *ex vivo* human skin permeation results and then the toxicokinetic results from the participants, separately, before discussing the coherence of the *in vitro* and participant results.

4.1. In vitro skin permeation

Phthalates are highly lipophilic substances and are thus, dependent on metabolism to permeate skin. The first step in phthalate metabolism is the hydrolysis of one ester group attached to the aromatic moiety (Fig. 1), which is performed by skin esterases (Sugino et al. 2017). Indeed, inhibiting skin esterases in experiments conducted with diffusion cells and *ex vivo* rat skin made the skin impermeable to skin exposure to dibutyl ester (Sugino et al. 2017). Previously frozen skin has less enzymatic activities, but skin esterases are still functional after freezing, albeit at a lower efficiency (Sugino et al. 2017). All our *ex vivo* human skin flow-through diffusion cells were able to metabolize the phthalates to the monoesters.

DBP and DEP emulsified in water permeated skin in higher concentrations compared to neat, with 39 % and 75 %, respectively, of the applied dose recovered in the receptor fluid as metabolites. The DEHP metabolites were quantified for the experiments where DEHP was applied neat to the skin but were not detected in any parts of the *in vitro* diffusion system when applied as an emulsion. This is contrary to the results found in our previous study (Hopf et al. 2014) where DEHP passed through human skin, detected as MEHP when emulsified in aqueous solution, and to a far lesser degree when applied neat to the *ex vivo* human skin. The difference between our current study and former was that we used human viable skin and this time previously frozen skin. Metabolism is known to be far less in previously frozen skin kept on saline solution for 24 hours compared to *in vivo* (Neil et al., 2020).

The solubility of the tested chemicals in the receptor fluid can be a limiting step in skin permeation experiments (OECD, 2004), especially for lipophilic substances such as phthalates. We wanted to understand the influence the amount of PEG in the receptor fluid had on sink conditions, and thus the ability of phthalate metabolites to cross the skin barrier. Highly lipophilic substances such as phthalates may be easily transported across the stratum corneum lipids, but will need to be metabolized to monoesters, which are relatively hydrophilic, to permeate the water-rich epidermis layer (Sugino et al. 2017). Phthalates are metabolism dependent for percutaneous absorption (Beydon et al., 2010; Sugino et al., 2017). The skin permeation rates (J) for all phthalates quantified as their monoester metabolites were low when phthalates were applied neat, irrespective of PEG concentrations in the receptor fluid (Table 2). This pattern was not observed for emulsified phthalates. DEHP did not permeate skin when emulsified and the DBP permeation rates were similar for both PEG concentrations (J: 0.38 versus 0.24 $\mu g/cm^2/h$) (Table 2). The picture was not as clear for emulsified DEP. We expected the skin permeation rate of emulsified DEP with 6 % PEG in the receptor fluid to be 15 times greater because of the dose increase (Table 1); however, the higher concentration of PEG in the receptor fluid increased the J to only being double. Recommendations according to our results are that if phthalate water emulsion is applied to the skin, then the receptor fluid should contain low amount of PEG. Our observation regarding the varying absorption rates for the different phthalates governed by their chain length supports previous studies (Chung et al., 2019; Hopf et al., 2014; Scott et al., 1987).

The vehicles in our study, water or neat, had an influence on the skin permeation of the phthalates. Phthalates emulsified in water had a faster rate compared to neat application. We applied 0.85 mL of emulsion to the skin surface of the *in vitro* skin experiments and water was still visible after 24 hours. Water decreases the skin's plastic extensibility caused by a reduction in the tissue's ability to hold water (Dhandapani et al., 2020) and this could potentially explain the difference in skin permeation rate.

We could only provide Kp for experiments that reached steady state (Table 2). We recommend future *in vitro* skin permeation studies with phthalates (especially the higher molecular weight phthalates) to increase the time to greater than 24 hours to account for possible skin storage. The relevance of leaving a substance on top of the skin more than 24 hours, which is outside of the OECD guidelines, is justified for phthalate exposures, we believe, because the stratum corneum (SC) absorbs the phthalate. A recent review (Gustafsson et al., 2020), found that the very low aqueous solubility and vapor pressure of many plasticizers, such as DEHP, did not necessarily limit exposure, because they have extensive binding and association with lipids. This would lead to an accumulation in the skin. Others have observed a phthalate accumulation in skin (e.g., Ng et al., 1992 used mice and pig full thickness skin with a receptor medium of 40 % ethanol and determined the parent compounds; Pan et al., 2014 used hairless guinea pigs and quantified both parent compounds and metabolites). We did not find evidence of accumulation in our metabolite mass-balance, but this might be because we only quantified the metabolites and not the parent compounds. We did not detect any secondary oxidative metabolites (Fig. 1) after DBP (3OH-MBP, 4OH-MBP, 3cx-MPP) nor DEHP (5OH-MEHP, 5oxo-MEHP, 5cx- MEPP, 2x-MEPP) exposures *in vitro* and therefore, based on the participant results discussed below, we conclude that these oxidation products are formed in viable human skin with active enzyme activity or elsewhere in the body, most likely the liver (Isobe et al. 2023).

When applied neat with 0.006 % of PEG in the receptor fluid, DEHP barely crossed the skin as MEHP and did not permeate when emulsified. This result was the inverse of our previous experiments with fresh skin (Hopf et al., 2014) where we found that DEHP, both neat and emulsified, crossed the skin as MEHP; but overall DEHP permeated skin poorly. Reported skin permeation parameters for DEHP using human skin vary greatly across three studies published (Barber et al., 1992; Hopf et al., 2014; Scott et al., 1987) and might be explained by experimental design (cadaver skin, heat separated, temperature ranges 32–37°C, and others). Our DEHP skin permeation rates were lower than previously reported (0.002 $\mu g/cm^2/h$ (<LOQ-0.005) in our study compared to literature ranges 0.0013–5.59 $\mu g/cm^2/h$) and this was also true for the permeation coefficient (Kp), which varied a few thousands fold ($0.00002*10^{-5}$ cm/h in our study compared to literature ranges $0.011*10^{-5}$ - $15.1*10^{-5}$ cm/h). These large variations in skin permeation parameters have several plausible explanations. Previously frozen skin was used in this study compared to the skin used in our earlier study where we mounted the skin less than two hours after it was removed from the patient. Previously frozen skin is less hydrated than fresh skin and therefore reacts differently to the oily neat DEHP compared to a small amount of DEHP in water.

DEP is the phthalate with the smallest molecular weight in our mixture and has the lowest hydrophobicity. MEP had the highest concentration of the three phthalates in the receptor fluid showing that DEP crossed the skin barrier readily compared to DBP and DEHP. Based on the results from experiments with 0.006 % PEG, we decided to run the second set of DEP experiments separately, with 6 % PEG in receptor fluid and a greater amount of DEP applied to the skin to increase the likelihood of reaching steady state. With a greater amount of DEP applied to the skin, MEP reached steady state when DEP was applied neat, but not when it was applied emulsified. Our skin permeation results should therefore be regarded as preliminary. DEP permeated less when applied neat compared to emulsified, irrespective of the amount of PEG (0.006 % or 6 % PEG) present in the receptor fluid, as water can disrupt the structure of the stratum corneum and enhance the absorption

of certain substances.

One explanation for the large differences in permeation we observed for DEP compared to DEHP is that the transepidermal routes for DEP are probably not the same for neat and in emulsion. In a recent review, an overview of the transepidermal micropaths were given (Barnes et al., 2021). In the transcellular route, the most direct path to permeate the skin, DEP would need to traverse the alternating layers of corneocytes and extracellular matrix composed of lipid lamellae, partitioning and diffusing into alternating hydrophilic and lipophilic domains. In the intercellular route, DEP would diffuse around the corneocytes and through the continuous lipid matrix. This is a long and a winding pathway. Small lipophilic molecules such as DEP applied neat would likely favor the transcellular route over the intercellular route. On the contrary, DEHP is highly lipophilic and would favor the longer intercellular route in either neat or in emulsion, as DEHP does not readily stay emulsified in water.

4.2. Participant study

Urinary elimination of MEP was fast with a mean apparent urinary elimination half-life of 8 hours (Fig. 3), which was longer than previously reported in another study (2.7 (0.7–11) hours (Krais et al., 2018)). The difference observed might be explained by type of exposures; Krais et al. 2018 exposed participants to aerosolized DEP droplets while wearing respirators (no inhalation exposures), while we applied neat substance directly onto the participants' skin. Interestingly, two of our participants had very long half-lives (13–14.5 hours) while the three others had half-lives from 3.8 to 6.6 hours. This could reflect inter-individual differences in oxidative metabolism of phthalates (Ito et al., 2014). Our urinary phthalate metabolite elimination results are similar to another study (Janjua et al., 2007), where a basic cream formulation containing DEP was applied to the whole body of 26 participants. They found that the urinary MEP concentration peaked 8–12 hours after application, which is in the same realm as our study (Fig. 3D).

The molar sum of two urinary DBP metabolites had maximum concentration at two time points: urine samples collected before the participants went to bed and the morning after (a flat line in the curve in Fig. 3E). Therefore, the calculated elimination half-life range was 2–9 h in urine, which is in line with other human data showing ranges from 2 to 4 h (Domínguez-Romero and Scheringer, 2019).

The DEHP metabolites eliminated in urine as MEHP, 5OH-MEHP, 5oxo-MEHP, and 5cx-MEHP, had apparent urinary elimination half-lives greater than 24 hours post-application to skin. Phthalate metabolite distributions vary depending on the route of entry (Krais et al., 2018; Liss et al., 1985; Peck and Albro, 1982). Ingested phthalates get into the blood from the intestines where phthalates are broken down by liver enzymes, known as the first pass effect. Phthalates absorbed through the lungs or across skin do not succumb to the first pass effect. Consequently, the phthalate monoesters are directly distributed into the blood after skin absorption and inhalation. Urinary metabolites were almost equally distributed at all times post-exposure, similar to what was observed after inhalation (Krais et al., 2018). We observed very low urinary DEHP metabolite concentrations and were unable to elucidate elimination kinetics. Furthermore, urinary DEHP metabolite concentrations increased after 24 hours. As opposed to ingestion that is transitory and intermittent, uptake of DEHP continuous for many hours, thus may contribute consequentially to the overall internal dose. We recommend that future studies of DEHP skin exposure collect 24-hour urine samples over multiple days.

4.3. Agreement of in vitro and participant results

A recent review gave an overview of the accumulating evidence that phthalates cross the skin barrier (Olkowska and Gržinić, 2022). Our results from the participant study exposed to phthalates and the *in vitro* flow-through diffusion cells support this review's conclusion. Our study also supports prior observations that phthalates with shorter alkyl chains have a greater skin permeation and shorter urinary elimination (Giovanoulis et al., 2020). This was true for *in vitro* experiments as well as among participants. The *in vitro* skin permeation experiments showed that dermal absorption was very slow and continued after 24 hours for DBP and DEHP experiments. This corroborated the urinary elimination kinetics that showed a continued rise in urinary phthalate metabolite concentrations after 24 hours post-application. Although, DEHP metabolites were not detected in any parts of the *in vitro* diffusion system, we observed urinary elimination kinetics for MEHP, 5OH-MEHP, 5oxo-MEHP, and 5cx-MEHP post-application on the participants' skin. This could potentially be explained by a deposit of DEHP in the stratum corneum and a slow release into the epidermis where it is metabolized. Using *ex vivo* skin directly harvested from the patients would have yielded results more comparable to those in the participants, but such experiments are difficult to execute in practice. The maximum urinary DEP, DBP, and DEHP concentrations were reached 11-, 17-, and 22-hours post-application, respectively. Taken together, the results in this study show that the flow-through skin permeation *in vitro* method with *ex vivo* human skin can be used to predict human absorption for very lipophilic compounds that require skin metabolism. This supports the results from a previous study that showed agreement between skin absorption results obtained *in vitro* and *in vivo* for hairless guinea pigs exposed to DEHP (Ng et al., 1992).

4.4. Study limitations

One limitation of our study was that we did not collect urine samples for a sufficiently long time after application to elucidate urinary elimination kinetics for the phthalate metabolites. Based on our findings, we suggest that urine should be collected for 48 hours post exposure and that the total volume for each urine void be measured. Another related limitation is that we did not collect flow-through diffusion samples after 24 hours in the *in vitro* experiments to reach steady state for the high molecular weight phthalates. Furthermore, we did not complete an *in vitro* mass balance as we were not able to measure the parent phthalate nor the unspecific metabolites. Overall, the strength of this study is that we used two approaches to further understand the importance of skin absorption of phthalates in humans. Furthermore, we used human skin in the *in vitro* experiments and human participants in the toxicokinetic study, and thus, were able to contrast the findings from the two approaches. We found that *in vitro* skin permeation experiments concurred well the observed *in vivo* results.

5. Conclusion

Our study showed that phthalate skin permeation decreased with increasing molecular weight among the three phthalates tested: DEP >DBP >DEHP. In vitro flow-through diffusion cell experiments with dermatomed human skin were efficient in predicting the slow urinary elimination observed in our participants. All the tested phthalates permeated skin as their respective monoester metabolite (*in vitro* data) and were further metabolized in the body before being eliminated in urine in participants (human participant data). Our *in vitro* study showed that phthalates emulsified in water permeated to a far greater extent compared to application of neat substances, except for DEHP which did not permeate in either condition. Despite this, participants exposed to neat phthalate mixture showed detectable skin absorption. Future studies should conduct *in vitro* skin permeation experiments for DEHP and other long-chain phthalates longer than 24 hours to increase the likelihood of reaching steady state. Even though phthalate exposures via the skin route contributes far less to the internal dose compared to inhalation and ingestion, we recommend that phthalate risk assessments include skin absorption to the total dose. Future developments would be to develop skin permeation *in situ* models for highly lipophilic

substances (logP >7) to better predict real life skin absorption.

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CRediT authorship contribution statement

Elena Reale: Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. **Aurélie Berthet:** Methodology, Validation, Writing – review & editing. **Claudia Pälmke:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Manon Benedetti:** Data curation, Validation, Writing – review & editing. **Myriam Borgatta:** Investigation, Methodology, Writing – review & editing. **Holger M Koch:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Nancy B Hopf:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft. **Hélène P. De Luca:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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