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Di-(2-propylheptyl) phthalate (DPHP) and its metabolites in blood of rats upon single oral administration of DPHP

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Abstract

Di-(2-propylheptyl) phthalate (DPHP) does not act as a reproductive toxicant or endocrine disruptor in contrast to other phthalates. Considering adverse effects of phthalates to be linked to their metabolism, it was the aim of the present study to investigate in the rat the blood burden of DPHP and its metabolites as a basis for understanding the toxicological behavior of DPHP. Rats were administered single oral doses of DPHP of 0.7 and 100 mg/kg body weight. Concentration-time courses of DPHP and metabolites were monitored in blood. The areas under the concentration-time curves in blood (AUCs), normalized for the dose of DPHP, showed the following order: DPHP < mono-(2-propyl-6-oxoheptyl) phthalate < mono-(2-propyl-6-hydroxyheptyl) phthalate = mono-(2-propylheptyl) phthalate < mono-(2-propyl-6-carboxyhexyl) phthalate (cx-MPHP). Glucuronidation of the monoesters accounted for less than 5% of total compounds. The elimination half-lives of the compounds ranged from 2.3 h (DPHP) to 8.2 h (cx-MPHP). The normalized AUCs of the metabolites were lower at the high dose of DPHP than at the low one indicating saturation kinetics of intestinal DPHP hydrolysis. The absence of toxicity to reproduction of DPHP may be related to the comparatively low bioavailability of the parent compound and its metabolites.

Abbreviations

AUC, concentration-time curve in blood calculated for $t \rightarrow \infty$; b.w., body weight; cx-MPHP(-d4), non- or ring-deuterated mono-(2-propyl-6-carboxyhexyl) phthalate; cx-MPHP, mono-(2-propyl-6-carboxyhexyl) phthalate; cx-MPHP-d4, ring-deuterated mono-(2-propyl-6-carboxyhexyl) phthalate; DEHP, di-(2-ethylhexyl) phthalate; DINP, di-isononyl phthalate; DPHP(-d4), non- or ring-deuterated di-(2-propylheptyl)

phthalate; DPHP, di-(2-propylheptyl) phthalate; DPHP-d4, ring-deuterated di-(2-propylheptyl) phthalate; MEHP, mono-(2-ethylhexyl) phthalate; MPHP(-d4), non- or ring-deuterated mono-(2-propylheptyl) phthalate; MPHP, mono-(2-propylheptyl) phthalate; MPHP-d4, ring-deuterated mono-(2-propylheptyl) phthalate; OH-MPHP(-d4), non- or ring-deuterated mono-(2-propyl-6-hydroxyheptyl) phthalate; OH-MPHP, mono-(2-propyl-6-hydroxyheptyl) phthalate; OH-MPHP-d4, ring-deuterated mono-(2-propyl-6-hydroxyheptyl) phthalate; oxo-MPHP(-d4), non- or ring-deuterated mono-(2-propyl-6-oxoheptyl) phthalate; oxo-MPHP, mono-(2-propyl-6-oxoheptyl) phthalate; oxo-MPHP-d4, ring-deuterated mono-(2-propyl-6-oxoheptyl) phthalate; $t_{1/2}$, half-life of the elimination phase

Keywords: Di-(2-propylheptyl) phthalate; metabolites; oral treatment; rat; blood

1. Introduction

Di-(2-propylheptyl) phthalate (DPHP), CAS No. 53306-54-0, is a high molecular weight branched phthalate ester. The technical product is marketed amongst others as “Palatinol®10-P“, which consists of about 99.5% phthalic ester. The alcohol moiety consists of 90% 2-propyl-heptanol and 10% 2-propyl-4-methylhexanol or 2-propyl-5-methylhexanol (Gries et al., 2012). The world-wide consumption of DPHP in 2012 was 208,000 metric tons (Schütze et al., 2015). DPHP is registered under REACH (Regulation (EC) No. 1907/2006) and intended as a plasticizer in PVC formulations. Commercial applications of DPHP include cables, carpet backing, car interiors, outdoor applications like pool liners, roofing membranes or tarpaulins, and also consumer products such as shoes and artificial leather (BASF, 2015; CPSC, 2011; NICNAS, 2003). Typical contents of the chemical in end-use products vary between 30-60% (w/w) (BfR, 2011; NICNAS, 2003). DPHP is used as a substitute for high molecular weight phthalates like di-isononyl phthalate (DINP) or di-isodecyl phthalate and for di-(2-ethylhexyl) phthalate (DEHP) which is under scrutiny due to toxicity to reproduction and endocrine activity.

Occupational exposure to DPHP may occur during production, packaging or cleaning of equipment (NICNAS, 2003). Recently it has been shown that also the general German population is exposed to DPHP due to its increased usage. The level of exposure for the general public ranges from 0.025 to 0.314 µg/kg b.w. per day (Schütze et al., 2015). Although DPHP is not intended for use in toys, food packaging or medical products (NICNAS, 2003), it has been detected in toys in contents of 10.1 - 48.2% (w/w) (BfR, 2011). The daily DPHP intake from such toys by children has been estimated to reach up to 135 µg/kg b.w. (BfR, 2011).

The metabolism of DPHP was studied in volunteers (Leng et al., 2014; Wittassek and Angerer, 2008). In analogy to other phthalates, the primary metabolite of DPHP is mono-(2-propylheptyl) phthalate (MPHP). MPHP is metabolized via omega and omega-1 oxidation, yielding mono-(2-propyl-6-carboxyhexyl) phthalate (cx-MPHP) and mono-(2-propyl-6-hydroxyheptyl) phthalate (OH-MPHP), respectively. The latter is further oxidized to mono-(2-propyl-6-oxoheptyl) phthalate (oxo-MPHP). All of these metabolites (Fig. 1) can be conjugated with glucuronic acid.

Toxicological data obtained in studies with rats suggest that DPHP is neither a reproductive toxicant nor an endocrine disruptor (BASF, 1995a, 2003, 2009; Furr et al., 2014). Following oral administration, increased liver weights and thyroid and pituitary effects are described, possibly in relation to a rat specific peroxisome proliferation (BASF, 1995b, 2009; Union Carbide, 1997, 1998). On the basis of the NOAEL of 40 mg/kg b.w. for subchronic toxicity in rats, an acceptable exposure for humans of 0.2 mg DPHP per kg b.w. per day has been derived (UBA, 2015). An oral reference dose of 0.1 mg/kg b.w. per day has been derived from the human equivalent 10% benchmark response level of 10 mg/kg b.w. per day for thyroid hypertrophy/hyperplasia in male adult rats (Bhat et al., 2014). Adverse effects of certain phthalates are related to metabolically formed monoesters (e.g. Foster et al., 1981; Oishi and Hiraga, 1980; Sjöberg et al., 1986). Regarding the species-specific burdens of the primary monoesters in venous blood, large species differences were identified in studies with DEHP (Kessler et al., 2004, 2012; Kurata et al., 2012; Rhodes et al., 1986). As a basis for a risk estimation of DPHP, it is therefore

reasonable to compare the burdens of the metabolically formed monoesters in blood of rats and humans. So far, no such data is available. Therefore, we investigated in the present study concentration-time courses of DPHP and its metabolites in venous blood of rats. The animals were orally administered two doses of 0.7 and 100 mg DPHP per kg b.w. in order to cover a large dose range. The low dose equals that used in a comparative study conducted with humans (manuscript in preparation).

2. Materials and methods

2.1. Chemicals

Standards of DPHP and its metabolites were used as non-deuterated or as ring-deuterated compounds. In the following, non-deuterated compounds are named DPHP, MPHP, OH-MPHP, oxo-MPHP, cx-MPHP and ring-deuterated compounds are named DPHP-d4, MPHP-d4, OH-MPHP-d4, oxo-MPHP-d4, cx-MPHP-d4. If it is not distinguished between non- and ring-deuterated compounds, the abbreviations are DPHP(-d4), MPHP(-d4), OH-MPHP(-d4), oxo-MPHP(-d4) and cx-MPHP(-d4), respectively.

Palatinol®10-P (purity 98%, GC analysis), DPHP-d4 (two batches: one with a purity of 84% according to GC analysis, the other one with a purity of >95% according to ^{13}C-NMR), MPHP (purity 90%, ^{13}C-NMR), and MPHP-d4 (two batches: one with a purity of 95% according to GC analysis, the other one with a purity of 75% according to ^{13}C-NMR) was supplied by BASF SE (Ludwigshafen, Germany). OH-MPHP, OH-MPHP-d4, oxo-MPHP, oxo-MPHP-d4, cx-MPHP and cx-MPHP-d4 were gifts from the Institute for Biomonitoring, Currenta (Leverkusen, Germany) and were synthesized at the Institut für Dünnschichttechnologie (Teltow, Germany). The indicated purities of the compounds were ≥95% (determined by ^{1}H-NMR).

Acetonitrile (Promochem picograde) was purchased from LGC Standards (Wesel, Germany) and water (LCMS grade) from Fisher Scientific (Loughborough, United Kingdom). Heparin-Natrium 25,000 I.E. ratiopharm was from Ratiopharm (Ulm, Germany) and beta-glucuronidase (E. coli K12) from Roche Diagnostics (Mannheim, Germany). All other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and were of highest purities available.

2.2. Animals and DPHP treatment

Male Wistar (Crl:WI(Han)) rats (250-280 g) were obtained from Charles River (Sulzfeld, Germany). This rat strain was also used in relevant toxicity studies (BASF 1995a, 1995b, 2003, 2009). The animals were housed in Macrolon® cages and provided with HEPA-filtered air in a TOP FLOW-IVC-system (Tecniplast, Buggugiate, Italy). The animal room was air-conditioned and the air was cleaned of particles by active charcoal filters. A constant light–dark cycle with light from 7:00 to 19:00 h was kept. Animals had free access to standard chow (Nr. 1324) from Altromin (Lage, Germany) and tap water.

DPHP was administered orally at a single dose of 100 or 0.7 mg/kg b.w. between 8 and 9 am. The experiments with 100 mg/kg DPHP were conducted prior to those with 0.7 mg/kg DPHP. For the high dose, DPHP (Palatinol®10-P) was administered. For the low dose, Palatinol®10-P could not be used because of background signals at the retention times of DPHP and its metabolites in blood of untreated rats (see 2.4. and the chromatograms (Supplementary Figure 1) shown in the supplementary material). Therefore, DPHP-d4 was used for the low dose. Both substances were given as aqueous emulsions in a saccharose solution (70% w/v) as vehicle. Emulsions of 10% (w/v) DPHP and 0.07% DPHP-d4 were prepared for the high and

the low dose, respectively. The test substance was administered by gavage using a graduated disposable syringe (1 ml) equipped with a stainless steel cannula. The animals received 1 ml emulsion per kg b.w. The actual dose was determined by reweighing the syringe and considering the purity of Palatinol®10-P and that of the DPHP-d4 batch used. Immediately after sacrifice of the rats with carbon dioxide, blood samples of 6-7 ml were collected from the vena cava inferior using disposable syringes (10 ml) rinsed with heparin. For each DPHP dose, 3 experiments were performed with collection of blood at time points of 0, 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0, 10 and 24 h after DPHP treatment. For each experiment, one calibration was done using blood from control animals.

2.3. Sample collection and preparation

Blood was transferred in aliquots of 0.6 ml to 9 Eppendorff caps (1.5 ml) each containing 60 µl acetonitrile or an internal standard solution. For the animals dosed with 0.7 mg/kg DPHP-d4, the internal standard solution consisted of DPHP (550 nmol/l) and MPHP, OH-MPHP, oxo-MPHP and cx-MPHP (each: 220 nmol/l) dissolved in acetonitrile. For the animals dosed with 100 mg/kg DPHP, the internal standard solution consisted of DPHP-d4 (550 nmol/l), OH-MPHP-d4, oxo-MPHP-d4 and cx-MPHP-d4 (each: 220 nmol/l) dissolved in acetonitrile; MPHP-d4 was lacking in this solution because it was not available at the time when the experiments with 100 mg/kg DPHP were performed. For the quantification of MPHP in blood, OH-MPHP-d4 and oxo-MPHP-d4 were used as substitutes for MPHP-d4 (see 2.5.). To each cap, 15 µl of an aqueous ammonium acetate solution (1.0 mol/l, pH 5.1) was added. The 9 caps were then subdivided into groups A, B, and C (3 caps each). To samples of groups A and B, 2 µl water, to samples of group C, 2 µl of beta-

glucuronidase were added. Before separation of the serum by centrifugation (18,000 x g, 10 min, room temperature), samples of groups B and C were incubated at 37°C for 30 min in a shaking water bath. From each cap, 250 µl serum was transferred to a 5-ml Falcon tube. After adding 500 µl water, 750 µl acetonitrile, 6 µl HCl (32%), and 1000 µl tert. butylmethyl ether, the tubes were shaken manually and vortexed. After centrifugation (5,340 x g, 10 min, 0°C), 1500 µl of the organic layer was transferred into a 2.2 ml Eppendorff cap. The solvents were evaporated at 40°C to dryness in a vacuum centrifuge (Univapo 100H equipped with a cold trap Unicyro 11C2L, UniEquip Laborgerätebau, Planegg, Germany). The residue was dissolved in 80 µl acetonitrile. Then, the samples were stored at -30°C until analysis.

For calibration, blood from control animals was added in aliquots of 0.6 ml to 9 Eppendorff caps (1.5 ml) each containing 60 µl of a solution of an analyte mixture in addition to the above described internal standards. When animals were dosed with 0.7 mg/kg DPHP-d4, the analyte mixture consisted of DPHP-d4, MPHP-d4, OH-MPHP-d4, and oxo-MPHP-d4 at final concentrations of 0, 2, 10, 25, and 50 nmol/l. The metabolite cx-MPHP-d4 was lacking in this mixture because no LC-MS/MS signal for this compound was obtained in the previously performed studies with 100 mg DPHP/kg b.w. (see 2.4.). When animals were dosed with 100 mg/kg DPHP, the analyte mixture consisted of DPHP at final concentrations of 0, 10, 100, and 1000 nmol/l and MPHP, OH-MPHP, oxo-MPHP, and cx-MPHP at final concentrations of 0, 20, 200, and 2000 nmol/l. Further sample preparation was performed as described above including splitting the blood samples into groups A, B, and C with 3 aliquots each.

2.4. High performance liquid chromatography tandem mass spectrometry

The LC-MS/MS system consisted of an HP1100 liquid chromatograph (Agilent, Waldbronn, Germany) and an API 4000 triple quadrupole mass spectrometer equipped with a turbo ion spray interface (Applied Biosystems, Darmstadt, Germany). The liquid chromatograph was equipped with a Luna C18 (2) column (150 mm × 2 mm i.d., 5 µm) obtained from Phenomenex, Aschaffenburg, Germany. Separations were carried out at a flow rate of 300 µl/min with sample volumes of generally 10 µl. An injection volume of 2 µl was used for samples when compound concentrations exceeded the calibration ranges (see 2.5). The mobile phase was 100% acetonitrile for the analyses of DPHP and DPHP-d4. For the analyses of the monoesters, the mobile phase consisted of 1% aqueous formic acid (solvent A) and acetonitrile (solvent B). The composition of the solvents at 0 min was 90% A and 10% B. Up to 15 min, the percentage of B increased linearly to 90% and remained constant up to 30 min. Within 1 min, the composition of the buffer was then adjusted back to 90% A and 10% B. The column was ready for a new injection after 36 min.

The turbo ion spray source of the API 4000 was operated at a temperature of 450°C in the positive ionization mode for the diesters and in the negative ionization mode for the monoesters at ion spray voltages of 5500 V and -4500 V, respectively. Nitrogen served as curtain (CUR = 10, all compounds), nebulizing (GS1 and GS2: 40 for diesters, 55 for monoesters), and collision gas (CAD: 10 for diesters, 11 for monoesters). The entrance potential was set on 10 V for the diesters and −10 V for the monoesters. The mass spectrometer was used in the multiple reaction-monitoring mode. The analyte specific parameters are summarized in Table 1. Peak areas of the signals were determined by means of the software Analyst 1.4.2 from Applied Biosystems. No LC-MS/MS signal could be obtained for cx-MPHP-d4 for unknown reasons. The retention times were: DPHP(-d4) 6.3 min, MPHP(-d4) 20.1

min, OH-MPHP(-d4) 17.7 min, oxo-MPHP(-d4) 17.2 min, and cx-MPHP 17.5 min. At these retention times, signals characteristic for the non-deuterated compound were detected in blood of control animals. Because the signals were absent in LC-MS-grade water (see Supplementary Figure 1), they most likely result from a common background of DPHP or of another phthalate with identical LC-MS/MS responses as DPHP and its metabolites. Accidental exposure can be excluded at the conditions of animal housing used.

2.5. Quantification procedure

Each of the 3 blood samples from groups A, B, and C, obtained from both the treatment experiments and the calibrations (see 2.3.), were analyzed once and the arithmetic means of the 3 aliquots were used for quantification. In the experiments with 100 mg DPHP/kg b.w. and in the corresponding calibrations, the arithmetic mean of the peak areas of OH-MPHP-d4 and oxo-MPHP-d4 was used as internal standard for the quantification of both MPHP and cx-MPHP. Linearity of the calibration curves is shown in Supplementary Figure 2. Samples with compound concentrations exceeding the calibration range were re-analyzed using 2 µl instead of 10 µl for LC-MS/MS analysis. In contrast to the deuterated compounds, the calibration curves of the non-deuterated compounds showed positive y-intercepts due to common background levels in rat blood.

Group A samples were used to calculate the blood concentrations of DPHP(-d4), group B samples those of free monoesters, and group C samples those of total (free + glucuronidated) monoesters.

The limits of quantification of the non-deuterated compounds defined as the corresponding common background levels in rat blood were calculated from the

mean y-intercepts of the three calibrations curves. They amounted to 40 nmol/l for DPHP, 150 nmol/l for MPHP, 25 nmol/l for OH-MPHP, 50 nmol/l for oxo-MPHP, and 5 nmol/l for cx-MPHP. The limits of quantification of the deuterated compounds - absent in blood of control rats - were defined as a signal-to-noise ratios of three. They averaged 1.0 nmol/l blood for DPHP-d4 and 0.1 nmol/l blood for MPHP-d4, OH-MPHP-d4, and oxo-MPHP-d4.

2.6. Kinetic analysis

The time courses of DPHP and its metabolites at both DPHP doses could be described using a biexponential function (1) $y = C_1 * e^{-\alpha t} + C_2 * e^{-\beta t}$. Fits to the data were done by means of Prism 6 (GraphPad Software, La Jolla, USA), assuming (2) $C_1 + C_2 = 0$. From the curve parameters, areas under the concentration-time curves for $t \rightarrow \infty$ (AUC) and half-lives of the elimination phases ($t_{1/2}$) were calculated:

(3) $AUC = C_1/\alpha + C_2/\beta$

(4) $t_{1/2} = \ln 2/\beta$

3. Results

Concentration-time courses of DPHP(-d4) and its monoester metabolites determined in blood are illustrated in semi-logarithmic plots (Figs. 2 and 3). The monoesters could undergo glucuronidation in contrast to DPHP. Their concentration-time courses are shown separately for the free compounds and the total (sum of free and glucuronidated) compounds (Fig. 3). At many time points, large variations of the concentrations of up to more than one order of magnitude were observed for all compounds and at both dose regimens. This may be at least in part due to the fact

that each time point represents an individual animal. About 1.5 hours after administration of 100 mg DPHP/kg b.w., its maximum concentration in blood is reached as deduced from the curve fit (Fig. 2A). At the low dose of 0.7 mg DPHP-d4/kg b.w., most time points were close to or below the detection limit (Fig. 2B). Therefore, no curve fit was possible. The dashed line in Fig. 2B was extrapolated from the curve fit of the high dose (Fig. 2A), assuming the kinetics of DPHP to be independent of the dose within the range studied. With the exception of the data points at ≥8 h, the curve matches most of the data points that are above the detection limits.

The maximum concentrations of the primary metabolite MPHP(-d4) and those of the secondary metabolites OH-MPHP(-d4) and oxo-MPHP(-d4) were reached after about one hour, independent of the DPHP(-d4) dose (Fig. 3). The peaks of cx-MPHP occurred about 2 hours later than those of the other metabolites. Among all compounds, MPHP(-d4) showed the highest maximum levels in blood at both dose regimens. OH-MPHP(-d4)- and cx-MPHP-levels were the second highest. The parent compound DPHP(-d4) showed the lowest maximum levels. During the elimination phase, parallel declines in blood were observed between free and total metabolites but not between the low and the high dose regimen (Fig. 3).

The declines in the lower dosed animals were steeper than those in the higher dosed ones. The corresponding $t_{1/2}$-values of the elimination phases, as calculated from the curve fits, are summarized in Table 2. At the high dose, $t_{1/2}$-values of the metabolites

were about 1.3-1.5 times longer than at the low dose. Among all compounds, DPHP showed the shortest $t_{1/2}$ of 2.4 h. MPHP was eliminated with a $t_{1/2}$ of 3.0 h. The $t_{1/2}$-values of OH-MPHP(-d4) and oxo-MPHP(-d4) were similar and were eliminated about 1.5 times slower than MPHP(-d4). The longest $t_{1/2}$ of 8.1 h was found with cx-MPHP.

A common measure of the blood burden of a compound is its AUC in blood. To enable a dose comparison, the AUC has to be normalized for the dose per kg b.w. Table 2 summarizes the normalized AUC values for DPHP(-d4) and its metabolites. AUCs of free metabolites contributed to at least 95% of the AUCs of total metabolites. The differences between low and high dose were very similar among the compounds varying by a factor of 2.5-2.9 with the exeption of DPHP(-d4) and cx-MPHP(-d4) because of lack of data at the low dose. The parent compound DPHP had by far the lowest normalized AUC value among all compounds. The most abundant compound was cx-MPHP with an AUC value of 137 nmol*h/l per µmol DPHP/kg b.w. The AUCs of MPHP and OH-MPHP were very similar and were about half of that of cx-MPHP. Oxo-MPHP showed the lowest AUC among the metabolites, being about 50% of those of MPHP or OH-MPHP.

4. Discussion

Primary and secondary phthalate monoesters formed metabolically from phthalate diesters can be excreted unchanged or as conjugates, e.g. glucuronides. According to our findings, monoesters formed from DPHP are scarcely glucuronidated in rat blood. It is unlikely that this results from saturation of the glucuronyl transferase activity, as has been discussed for other phthalate monoesters (Silva et al., 2011), because the low proportion of conjugated DPHP metabolites was independent of the

dose of DPHP. Also, the absolute metabolite concentrations in rat blood at the lower DPHP dose were rather small (below 100 nmol/l).

Upon oral intake of DPHP, its normalized AUC was low in comparison to the AUCs of the metabolites and amounted to about 4% of the sum of the AUCs of all compounds (high dose). Such a low bioavailability in rats has been observed also with orally ingested DEHP (Kessler et al., 2004; Kurata et al., 2012; Pollack et al., 1985; Teirlynck and Belpaire, 1985).

It has not been investigated which tissue plays the predominant role in the first pass metabolism of DPHP after its oral administration. According to investigations in vitro with the structural homologue DEHP, the hydrolytic activity is particularly high in the small intestine of rats (Albro and Thomas 1973; Rowland 1974). Therefore, and because the AUC of DPHP was very small, we conclude that the intestine represents the major site of initial metabolism of DPHP.

Metabolic elimination of MPHP from the blood occurs via omega oxidation to cx-MPHP or omega-1 oxidation to OH-MPHP that is further oxidized to oxo-MPHP. The comparison of the AUC of cx-MPHP and the summed AUCs of OH- and oxo-MPHP suggests equal effectiveness of both pathways for the metabolic elimination of MPHP.

The question arises whether the kinetics of the metabolic elimination of MPHP is dose dependent. At concentrations of MPHP far below saturation of its metabolism, half-life of MPHP and the ratios of its AUC to those of its metabolites should not increase substantially with the DPHP dose. Both hold true with respect to the omega-1 oxidation of MPHP. Regarding the omega oxidation of MPHP, a corresponding comparison cannot be made because data of cx-MPHP are lacking at the low dose. Also the oxidation of OH-MPHP is not saturated because the ratios of

half-lives and AUCs of OH-MPHP to oxo-MPHP are almost identical at both DPHP doses. Nevertheless, the DPHP dose-dependent increases in $t_{1/2}$ of OH-MPHP and oxo-MPHP are larger than that of MPHP. This cannot be attributed to the metabolism of the secondary metabolites, because oxo-MPHP is not further biotransformed as concluded from findings with DEHP (Albro and Lavenhar 1989). Possibly, the excretion of these metabolites follows saturation kinetics.

The normalized AUCs of each DPHP metabolite are 2.5-2.9 fold lower at the high DPHP dose (100 mg/kg b.w.) as compared to the low one. This results probably from saturable intestinal formation of MPHP from DPHP as can be deduced from findings with other phthalates. Such a saturation was concluded for DINP upon repeated oral administration of doses higher than 250 mg/kg/day to rats (Clewell et al. 2013). For DEHP, saturation kinetics of intestinal hydrolysis has been shown in vitro using contents of the small intestine from rats (Rowland et al., 1974). Albro et al. (1982) investigated the appearance of DEHP in livers of rats following oral administration of up to 1000 mg/kg DEHP. The authors reported an absorption threshold of 450 mg/kg DEHP suggesting saturation of the intestinal hydrolysis of DEHP at oral doses of ≥450 mg/kg. Thus, the phthalate dose leading to saturation of intestinal hydrolysis seems to be dependent of the chain lengths of the alcohol residue: the longer the chain, the lower the saturation dose.

The activities of the intestinal lipases towards DPHP seem to be lower than those towards DEHP, because the sum of the normalized AUC values (nmol*h/l per µmol phthalate/kg b.w.) of the DPHP metabolites (≥477 at 0.7 mg DPHP/kg b.w.; 328 at 100 mg DPHP/kg b.w.; sum of total compound-specific values given in Table 2) is lower than that of the metabolites of DEHP (≥695 at 30 mg DEHP/kg b.w., Kessler et al. 2004). This is in accordance with the findings that intestinal hydrolysis of

phthalates decreased with increasing length of the alcohol moiety (Lake et al., 1976; Rowland et al., 1977).

The present data together with data on other phthalates suggest that the kinetics of orally applied phthalates in rats at doses at which the elimination of the primary metabolite is not saturated depends largely on the hydrolysis of the diester in the intestine. This relationship has a great impact on the systemic burdens of potentially effective monoesters and thus could be one reason why phthalates with straight chain lengths of the alcohol moieties of C_3-C_7 (total C of the alcohol ≤ 8) were reproductive toxicants in contrast to those with longer straight chains (Furr et al., 2014; Gray et al., 2000; Poon et al., 1997). Since the kinetics of phthalates can be species-specific (shown for DEHP: Kessler et al., 2004, 2012; Kurata et al., 2012; Rhodes et al, 1986), the present data set in rats will be compared with the outcome of current investigations in humans in order to support the risk assessment of DPHP.

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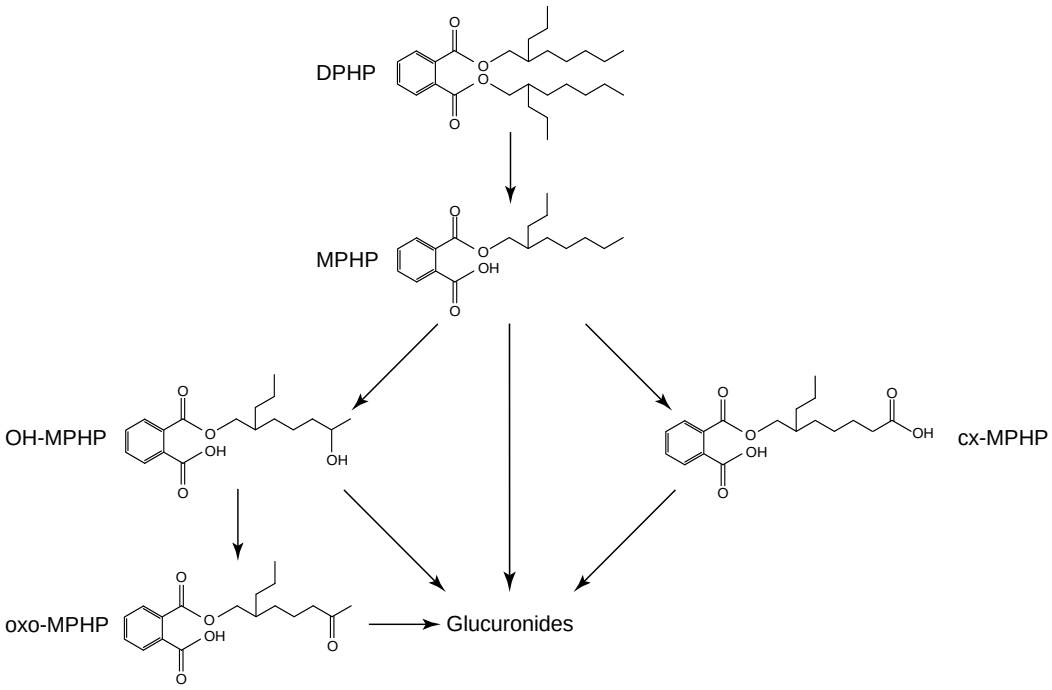

Fig. 1. Pathway of DPHP to metabolites investigated in the present study

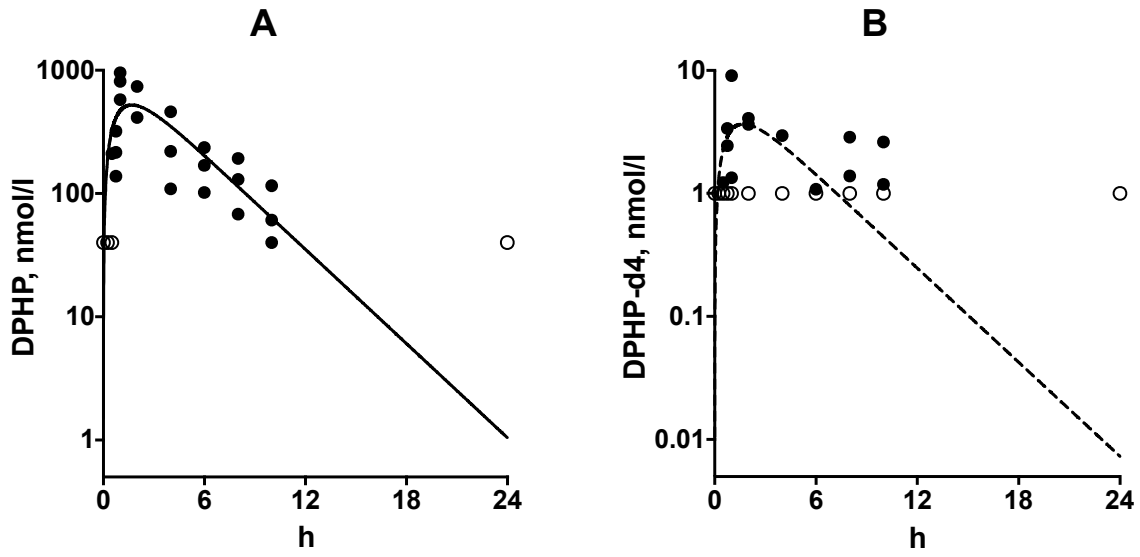

Fig. 2. Concentration-time courses of DPHP(-d4) in blood of rats upon single oral administration of (A) 100 mg DPHP/kg b.w. or (B) 0.7 mg DPHP-d4/kg b.w. Symbols: measured data in individual rats; open symbols: limit of quantification (LOQ); solid line: curve fitted to values higher than the LOQ; dashed line: predicted from the curve obtained for the dose of 100 mg DPHP/kg b.w. by using the same rate constants. The biexponential functions describing the curves are given in the Addendum to Fig. 2 in the supplementary material

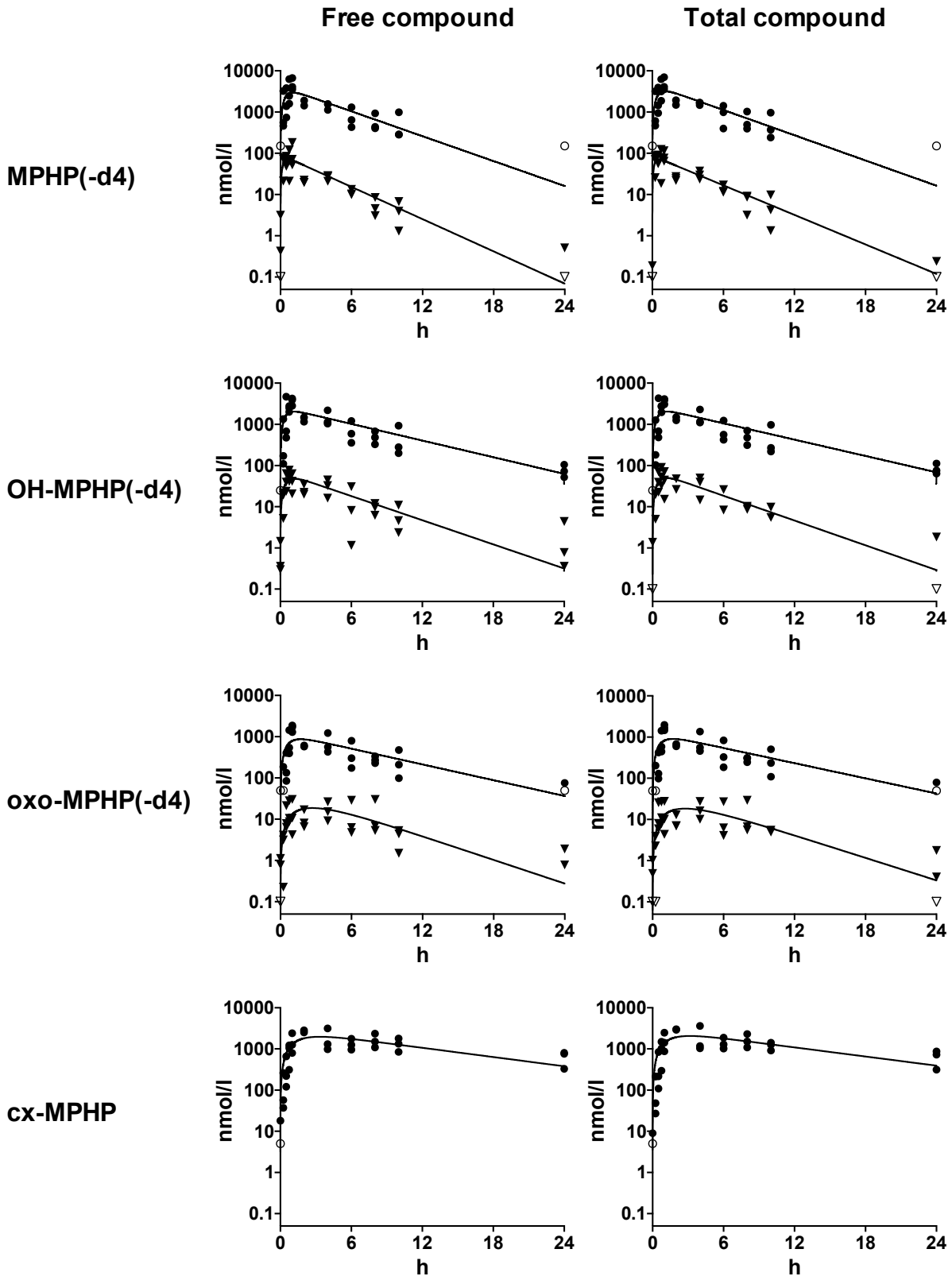

Fig. 3. Concentration-time courses of DPHP(-d4) metabolites in blood of rats upon single oral administration of 100 mg DPHP/kg b.w. (circles) or 0.7 mg DPHP-d4/kg b.w. (triangles). Free compounds are unconjugated metabolites; total compounds

are the sum of free and glucuronidated compounds. Filled symbols: measured data in individual rats (mean values of 3 determinations); open symbols: limit of quantification (LOQ); lines: curves fitted to values higher than the LOQ. The biexponential functions describing the curves are given in the Addendum to Fig. 3 in the supplementary material

Table 1: Compound-specific MS/MS parameters

Analyte*	Parent ion	Daughter ion	DP [V]	CE [V]	CXP [V]
DPHP	447	149	40	37	26
DPHP-d4	451	153	40	27	12
MPHP	305	134	-60	-24	-7
MPHP-d4	309	130	-60	-24	-7
OH-MPHP	321	121	-65	-28	-5
OH-MPHP-d4	325	125	-40	-22	-11
oxo-MPHP	319	121	-55	-28	-7
oxo-MPHP-d4	323	125	-65	-28	-5
cx-MPHP	335	187	-40	-22	-11

DP: declustering potential; CE: collision energy; CXP: cell exit potential; * d4 indicates ring-deuterated compounds

Table 2: Areas under the concentration-time curves and half-lives of the elimination phases of DPHP(-d4) and its metabolites in blood of rats upon single oral administration of DPHP(-d4)

Compound	Half-life [h][a]				AUC [nmol*h/l per µmol DPHP(-d4)/kg b.w.][b]			
	total compound[c]		free compound		total compound[c]		free compound	
	low dose[d]	high dose[e]	low dose[d]	high dose[e]	low dose[d]	high dose[e]	low dose[d]	high dose[e]
DPHP(-d4)	-	-	n.d	2.4	-	-	n.d	13
MPHP(-d4)	2.5	3.0	2.3	3.0	194	78	185	73
OH-MPHP(-d4)	3.0	4.6	3.1	4.5	183	72	179	70
oxo-MPHP(-d4)	3.3	4.9	3.2	4.7	100	36	99	34
cx-MPHP	n.d.	8.2	n.d.	8.1	n.d.	142	n.d.	137

[a] derived from the exponential functions fitted to the data presented in Figs. 2 and 3

[b] area under the concentration-time curve calculated for $t \rightarrow \infty$, normalized for the DPHP dose as derived from the exponential functions fitted to the data presented in Figs. 2 and 3

[c] sum of free and glucuronidated compound

[d] 0.7 mg DPHP-d4 per kg b.w.

[e] 100 mg DPHP per kg b.w.

n.d.: not determined