



Dermal penetration and resorption of beta-naphthylamine and N-phenyl-beta-naphthylamine from lubricants in an *ex vivo* human skin model



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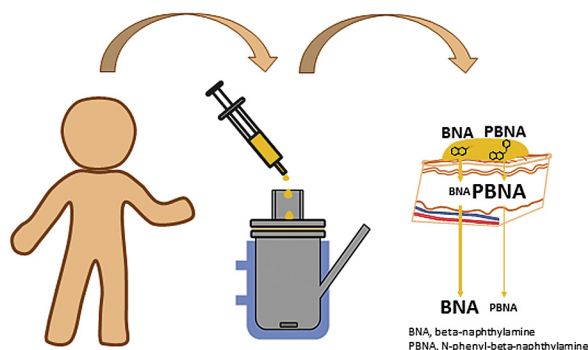
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HIGHLIGHTS

- New insights into dermal uptake of environmental and occupational relevant AA's.
- Workplace exposure scenarios for investigation of dermal resorption of AA's *ex vivo*.
- For the first time intra- and trans-dermal penetration of PBNA has been shown.
- Characterization of dermal penetration of carcinogenic BNA through human skin.
- Formulation and co-exposure have a relevant impact on dermal uptake of both AA's.

GRAPHICAL ABSTRACT



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ABSTRACT

Dermal Penetration of aromatic amines (AA's), often suspected or known to be carcinogenic, can play an important role in the overall human exposure. However, information on penetration of certain AA's is poor and inconsistent. Penetration of the former lubricant additive N-phenyl-beta-naphthylamine (PBNA) and its contaminant beta-naphthylamine (BNA) a known carcinogen was investigated and the influence of formulation and co-application characterized.

Percutaneous penetration of BNA and PBNA through freshly excised human skin ($n = 8$; 48 h) was investigated using an *ex vivo* diffusion cell model. Both AA's were applied in a technical-conform lubricant or dissolved in hexane. The amount of BNA and PBNA applied to skin was 0.52 and 259 $\mu\text{g}/0.64 \text{ cm}^2$. The analytical determination of AA's was performed by GC-MS.

Both, BNA and PBNA penetrated through human skin (38 vs. 5% of applied dose). In contrast to BNA, the percutaneous penetration of PBNA continued beyond the end of exposure. Co-exposure of both AA's increased the intradermal uptake of BNA and PBNA ($p < 0.05$). Exposure in lubricant showed the least overall penetration (2.9 and 1.9% of applied dose).

Abbreviations: AA's, aromatic amines; BNA, beta-naphthylamine; CAS, chemical abstract service; CV, coefficient of variation; GC-MS, gas chromatography-mass spectrometry; HCl, hydrochloric acid; LOD, limit of detection; LogP, decadic logarithm of the octanol/water partition coefficient; NLGI, national lubricating grease institute; PBNA, N-phenyl-beta-naphthylamine; SD, standard deviation; SEM, standard error of the mean.

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1. Introduction

Aromatic amines (AA's) are widely used in the production of rubber, dyes, pesticides or pharmaceuticals. Additionally, they are components of coal tar and pitch. Aromatic amines can be released into the environment by combustion of wood and fuel or from tobacco smoke. Since the majority of AA's is classified as carcinogenic for humans and/or animals (DFG, 2016) this group of substances is of concern for environment and human health and particularly for occupational health.

In the past, *N*-phenyl-beta-naphthylamine (PBNA; *N*-phenyl-naphthalen-2-amine, CAS 135-88-6) was often implemented among others as antioxidant for lubricating oils and greases (Falbe and Regitz, 1991) in the rubber industry and other industrial workplaces. However, as a result of the manufacturing process PBNA was contaminated with beta-naphthylamine (BNA, naphthalen-2-amine, CAS 91-59-8) up to a concentration of 1000 mg/kg (DGUV, 2014). It has long been known that BNA can cause bladder cancer in humans which is the reason why it was substituted in the first place by the supposedly non-hazardous PBNA. However, based on data from animal studies, there are indications that PBNA too might be carcinogenic (DFG, 2016). Therefore the handling of contaminated lubricants may be associated with a considerable health risk for these workers.

Along with inhalation of vapors and dusts, skin contact is a common route of occupational exposure to a multitude of chemicals. For several AA's a substantial percutaneous penetration through animal (Baynes et al., 1996; Levillain et al., 1998) and human skin (Yourick and Bronaugh, 2000; Kenyon et al., 2004; Korinth et al., 2008) is confirmed. With 54% of the applied dose, a relevant penetration of BNA through human skin was demonstrated *ex vivo* (Lüersen et al., 2006). For PBNA qualitative and quantitative data on percutaneous penetration is scarce. In a diffusion cell study no penetration of PBNA through excised human skin was observed within an observation period of 24 h (Wellner et al., 2008). However, the study provides no information regarding the intradermal situation. Considering its high lipophilicity (LogP_{OW} 4.38) it is possible that PBNA will be retained in skin as well as its passage through skin will be prolonged.

The use of an *ex vivo* model for the measurement of absorption of AA's in and through human skin is essential since *in vivo* human studies cannot be implemented for carcinogenic substances. It is widely known that results obtained from animal studies are only to a limited extent transferable to humans (Jung and Maibach, 2015) due to differences in the epidermal barrier. Compared to this, the epidermal barrier function of freshly excised human skin *ex vivo* resembles *in vivo* conditions best.

The first aim of the present study was to establish an *ex vivo* penetration model for the study of dermal penetration of AA's. With this model, the dermal penetration (uptake into a certain layer or structure i.e. stratum corneum or deeper structures) and the resorption (uptake into systemic circulation or into its equivalent, the receptor fluid) characteristics of BNA and PBNA from a typical workplace lubricant as well as during and after direct, i.e. sole or mixed exposure, in and through freshly excised human skin were investigated.

2. Materials and methods

2.1. Test compounds and their physicochemical properties

Beta-naphthylamine and *N*-phenyl-beta-naphthylamine with a purity of 98 and 97%, respectively, were used for percutaneous penetration experiments. The aromatic amines (AA's) were purchased from Sigma Aldrich (Steinheim, Germany). With <0.0024%, PBNA used for experiments offered the lowest BNA impurity among different commercial suppliers (Weiss et al., 2013). The physicochemical properties of test compounds are shown in Table 1. Data were obtained from the PhysPro[®] Database of Syracres Research Corporation (SRC, Syracuse, NY, USA; <http://www.syrres.com>).

2.2. Preparation of skin membranes

Freshly excised human skin was used for percutaneous penetration experiments. Skin from the abdominal area of 2 female donors (44 and 73 years) was obtained anonymously from a local clinic immediately following abdominoplasty. The study was performed according to ethical guidelines of our university and patients gave written informed consent. After removing the subcutaneous fat tissue the skin was prepared to a thickness of ~0.9 mm using a scalpel. Skin integrity was assessed visually prior to mounting the skin on diffusion cells by scanning the surface for macroscopic anomalies of skin structure, e.g., scars, striae or holes. Following equilibration for about 30 min, skin surface temperature was measured by a digital precision thermometer (GMH 1160 with GOF 500 universal probe, type K; Greisinger electronic GmbH, Regenstauf, Germany).

2.3. Preparation of test solutions and dermal application of lubricant

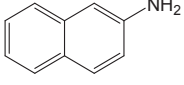
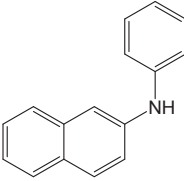
Industrial lubricant (calcium soap grease, NLGI consistency number 1), spiked with BNA and PBNA at a dose of 20 mg/kg (0.002%) and 10 g/kg (1%), respectively, was produced by and purchased from Zeller + Gmelin GmbH & Co. KG (Eislingen, Germany). The viscous lubricant was filled in a 1 ml syringe (Omnifix[®] – F 1 ml; B. Braun, Melsungen, Germany) and centrifuged (1 min, 1500 rpm to remove air bubbles). An amount of 0.035 g of the lubricant was squeezed on the skin fixed in diffusion cells (~40 µl), and distributed evenly with an embossing tool (Ø 5 mm). In preliminary tests (n = 9) the weight difference of the applicator before and after spreading the lubricant on the skin surface was measured with an analytical precision balance (XA105DU; Mettler Toledo[®], Giessen, Germany) and the final concentration of lubricant applied to skin calculated (mean ± SEM: 0.026 ± 0.0005 g/0.64 cm²).

According to these results, solutions of BNA (4.1 µg/l) and PBNA (2023.4 µg/l) in hexane were prepared for single, as well as for mixed exposure.

2.4. Percutaneous penetration experiments

Percutaneous penetration of BNA and PBNA was investigated using static PermeGear[®] diffusion cells (flat flange joint vertical system; 9 mm orifice; exposure area 0.64 cm²; receptor chamber

Table 1
Physicochemical properties of the test compounds.

Test compound [CAS number]	Chemical structure	Molecular weight (g/mol)	Water solubility (g/l)	LogP ^a	Physical form at RT ^b (Melting point, °C)	Boiling point (°C)
beta-Naphthylamine [91-59-8]		143.19	0.189	2.28	Solid (113.0)	300.0
N-Phenyl-beta-naphthylamine [135-88-6]		219.29	0.006	4.38	Solid (108.0)	395.5

^a LogP is the decadic logarithm of the octanol/water partition coefficient.

^b RT = room temperature.

volume ~5 ml) (SES GmbH, Bechenheim, Germany), which are similar to the cells described by Franz (1975). For receptor fluid (as equivalent of the systemic uptake), Ringer's solution (Serumwerk Bernburg AG, Bernburg, Germany) was mixed with 20% human albumin (CSL Behring AG, Bern, Switzerland) (75:25 v/v) and was titrated with 8.4% sodium bicarbonate solution to a pH of 7.4. The receptor chambers were filled with receptor fluid, supplied with a magnetic Teflon[®] stirring bar, and heated up to 35 °C during the experiments by a thermostatic circulating water bath (MV-4; Julabo GmbH, Seelbach, Germany).

To control for background contamination, blank samples of 1 ml receptor fluid were taken from each diffusion cell before the skin was exposed to the test compounds. The sampled volume was immediately replaced by new receptor fluid. Percutaneous penetration experiments were performed using 4 skin membranes in parallel from each donor. Exposure to BNA and PBNA was performed either directly as sole or mixed exposure in hexane (128 µl; 200 µl/cm²) or through application as mixture in the lubricant to the epidermal side of the skin fixed between donor and receptor chamber of diffusion cells. After n-hexane application the solvent evaporated completely within 30 min. In the different exposure scenarios equal concentrations of BNA and PBNA were applied to the skin (0.52 µg/0.64 cm² and 259 µg/0.64 cm²).

After 8 h, skin surfaces were gently wiped with one single dry cotton swab each to remove the excess of the AA's. Due to the viscosity and stickiness, 3 dry cotton swabs were used for skin samples exposed to lubricant. The cotton swabs were transferred into flange glass vials containing 4 ml of hexane and stored frozen at -20 °C until analysis. During the course of experiments, the exposure chambers of diffusion cells were left open.

Receptor fluid samples (1 ml) were collected at defined points in time after start of the experiments, namely during (2, 4, and 8 h) and after (16, 24, and 48 h) exposure of AA's. The sampled volume was immediately replaced by new receptor fluid.

To determine the reservoir capacity of the stratum corneum, 10 consecutive tape strips were torn off from each exposure area at the end of experiments, preserved in closed flange glass vials and stored frozen. Prior to analysis, the tapes were digested in 4 ml hexane for 90 h at room temperature. The first strip (strip 1) was preserved separately from the following 9. For skin exposed to lubricant the initial, non-sticky "pre-strips" were discarded, since they did not remove stratum corneum but the remaining layer of lubricant. The first tape strip sticking to skin was declared as "stratum corneum removing" strip 1. This strip and the following 9 strips were preserved as described above. Strip 1 was not included in the calculation of skin penetration into the stratum

corneum. Following the tape stripping procedure, skin punches (ø 10 mm) were taken from the exposure areas and weighed using a precision balance (XA105DU; Mettler Toledo[®], Giessen, Germany). The samples were preserved in closed flange glass vials and stored frozen at -20 °C until analysis. Directly prior to analysis, skin samples were digested in 2.4 ml aqueous HCl (37%) solution for 2.5 h in the heating stove.

2.5. Chemical analyses

Receptor fluid and digested skin samples were analyzed for their content of BNA and PBNA using a GC-MS system. Technical details and a precise description of the method can be found in Zobel et al. (2018). Table 2 shows the analytical parameters for the detection of aromatic amines. The calibration was performed with matrix-matched standards and the precision for BNA and PBNA was determined by analyzing matrix spiked with two concentration levels ($Q_{low} = 5.09$ and 10.3 µg/l for receptor fluid and tape strips, 50.9 and 103 µg/l for skin punches; $Q_{high} = 509$ and 1029 µg/l for receptor fluid and tape strips, 209 and 1029 µg/l for skin punches, respectively). The limit of detection (LOD) was assessed according to DIN32645.

2.6. Data calculation and statistics

Percutaneous penetration kinetics of BNA and PBNA were calculated from the concentrations of the AA's in the individual receptor fluid samples. Data were corrected for the dilution resulting from the sampling procedure. Missing values (5.1%) were replaced by the product of the mean percentage change in

Table 2
Analytical data for the detection of aromatic amines (Zobel et al., 2018).

Compartment	Test compound	Calibration	LOD ^c	CV _L ^d	CV _H ^e
		(µg/l)	(µg/l)	(%)	(%)
Tape Strips	BNA ^a	1.0–494	0.02	14.2	3.3
	PBNA ^b	2.0–1018	0.07	14.1	5.9
Skin punches	BNA	1.0–507	0.09	4.1	11.5
	PBNA	2.1–1029	1.36	9.5	6.4
Receptor fluid	BNA	1.0–507	0.02	5.2	3.8
	PBNA	2.1–1029	0.03	7.1	9.1

^a BNA: beta-naphthylamine.

^b PBNA: N-phenyl-beta-naphthylamine.

^c LOD: limit of detection.

^d CV_L: coefficient of variation for low concentration level.

^e CV_H: coefficient of variation for high concentration level.

penetration (within the same exposure group) and the previous value. Percutaneous penetration rates of BNA and PBNA are given as maximal fluxes, which are calculated from the linear part of the slope of cumulative penetrated amounts per area of skin surface and time ($\mu\text{g cm}^{-2} \text{h}^{-1}$). The times it takes for the AA's to penetrate through skin (break-through times) are the intersections of the extension of the linear part of these slopes with the time (X) axes. Dermal distribution patterns of BNA and PBNA, calculated for every single diffusion cell, are given as ratio. If not indicated otherwise, data are presented as mean values with standard errors of the means (SEM).

Percutaneous penetration kinetic data were compared by analysis of variance (one-way ANOVA). Differences between the various exposure scenarios in the amounts of AA's penetrated in the receptor fluid as well as in the stratum corneum and in the epidermis/dermis were compared by Mann-Whitney-U-Test (SPSS® 21.0 for Windows; SPSS Inc., Chicago, IL, USA). As level of significance $p < 0.05$ was chosen for all results.

3. Results

The close range in skin surface temperature of 32.3–33.3 °C (means \pm SD: 32.8 \pm 0.28 °C) indicates similar exposure conditions for all experiments. Visual examination did not show lesions of the skin membranes. The weight differences of the applicator before and after spreading lubricant on the skin surface (means \pm SD: 0.0083 \pm 0.0012 g) indicate that equivalent amounts of BNA (means \pm SD: 0.54 \pm 0.025 μg) and PBNA (means \pm SD: 268.8 \pm 12.5 μg) were applied in lubricant compared to direct application in hexane (0.52 μg vs. 259 μg). Blank receptor fluid samples did not indicate background contamination with AA's (data not shown). The highest concentrations of BNA and PBNA in the receptor fluid samples (40.7 and 949 $\mu\text{g/l}$) were clearly within their solubility limits in aqueous media.

The penetration kinetics of BNA and PBNA are shown in Fig. 1. Already within the exposure period (8 h) 82–92% of cumulative penetrated amount passed through the skin for BNA. Thereby, the transdermal penetration of BNA reached its maximal flow rate within the first 4 h in all test series. In contrast, only 7–15% of the total cumulative amount of PBNA penetrated within the first 8 h and penetration continued beyond termination of exposure. Also, maximal flow rate was not reached within the exposure period. A completion of PBNA penetration was not observed up to the end of experiments (48 h). Following exposure in lubricant, the penetration profiles were similar but the cumulative penetrated amounts of BNA and PBNA considerably lower (by ~90 and ~50%, respectively; $p < 0.05$) compared to both direct exposure scenarios.

Percutaneous penetration parameters for BNA and PBNA are summarized in Table 3. Solely applied, BNA appeared within ~38 min in the receptor fluid. Applied as mixture with PBNA in hexane or lubricant this break-through time was shortened by about factor 3.5 ($p < 0.05$) and 2.9, respectively. PBNA by contrast, had a break-through time of about 4 h for sole and mixed application in hexane and >7 h for mixed exposure in lubricant. Maximal flux was lowest for both AA's when exposed in lubricant compared to the direct test scenarios ($p < 0.05$).

Considering the intradermal distribution of the AA's at the end of the experiment (48 h), BNA accumulated evenly in stratum corneum and epidermis/dermis after sole exposure (0.006 vs. 0.0051 μg). Mixed exposure together with PBNA led to a slight shift of intradermally accumulated BNA towards epidermis/dermis. When exposed in lubricant, significantly smaller amounts of BNA were detected in the epidermis/dermis, while no BNA was detected in the horny layer. With sole application of PBNA in hexane, a ~5-fold higher amount was detected in the stratum corneum compared to

the epidermis/dermis. The amounts in stratum corneum and epidermis/dermis were higher (factor 1.9 vs. 4.3, $p < 0.05$) when PBNA was applied together with BNA. The PBNA skin reservoir in the horny layer was lowest for the exposure of the AA's in lubricant ($p < 0.05$).

The transdermally penetrated amounts of AA's in relation to the amounts detected in the skin (stratum corneum + epidermis/dermis) at the end of experiments (48 h) are shown in Fig. 2. With a ratio of 17.9 the vast bulk of penetrated BNA was detected in the receptor fluid, while PBNA preferably accumulated within the skin after sole application (ratio: 0.62). Although the ratio for BNA shifted towards skin depot when applied together in hexane or in lubricant (by 31 or 81%), it stayed well above 3. The distribution pattern of PBNA fluctuated around 1 when co-applied with BNA (i.e. equal amounts found in receptor fluid and skin depot). With neat exposure, more PBNA was found in the skin (ratio: 0.34) and less after exposure in lubricant (ratio 1.91).

4. Discussion

Though the uptake of hazardous substances by inhalation is a common exposure route, it often does not represent the main pathway into the organism. Percutaneous penetration of some AA's is known to contribute considerably to the overall exposure (Kenyon et al., 2004; Korinith et al., 2007) or may even exceed the uptake by inhalation (Korinith et al., 2013) in dependency on the exposure scenario. Therefore, evaluating the dermal route of exposure to AA's might be crucial to assess health hazards. However, estimating the dermal uptake of BNA and PBNA in humans is difficult due to the lack of data. The present study explored the dermal uptake of AA's using the model substances BNA and PBNA with a special focus on occupational exposure conditions and co-exposure.

Since data obtained from *ex vivo* diffusion cell studies using excised human skin correlated significantly with human *in vivo* data (Lehman et al., 2011) it has proven to be a valuable model investigating the penetration of potential carcinogenic and/or toxic substances in human skin. The experimental protocol used in the present study is consistent with guidelines for investigating dermal absorption of chemicals *ex vivo* (WHO, 2006; OECD, 2011). For frozen storage of skin no adverse effects on the percutaneous penetration were reported for several chemicals up to a LogP_{OW} of 2.1 (Nielsen et al., 2011; Dennerlein et al., 2013). However, since an increase in dermal penetration through human and rat skin after freezing was reported for highly lipophilic substances (Babu et al., 2003; Swarbrick et al., 1982), freshly excised human skin was used in the present study. Integrity of applied skin membranes can be assumed since all experimental data show low variances and were within three standard deviations of the mean (Howes et al., 1996). The industrial production of the lubricant spiked with the AA's as used in the present study guaranteed that it is equivalent to commercial work-place formulations and ensured an even distribution of the AA's within the lubricant. An occupational worst-case scenario was simulated by the applied amounts of AA's matching the maximal values reported in lubricants in the 1950s and 1960s (DGUV, 2014) and the exposure duration of 8 h, matching a full work shift. To maximize the time BNA and PBNA are in direct dermal contact, hexane which evaporated within 30 min of exposure, was chosen. Considering that with a calculated epidermal desquamation rate of $1 \times 10^{-9} \text{ cm/s}$ (Kimura et al., 2012) the outermost layer of the stratum corneum exfoliates within one day, the amounts of substance extracted from the first tape strip were not included in the calculations of the total absorbed amounts.

About 35% of the applied amount of BNA penetrated within 48 h through human skin, which is in agreement with findings of a

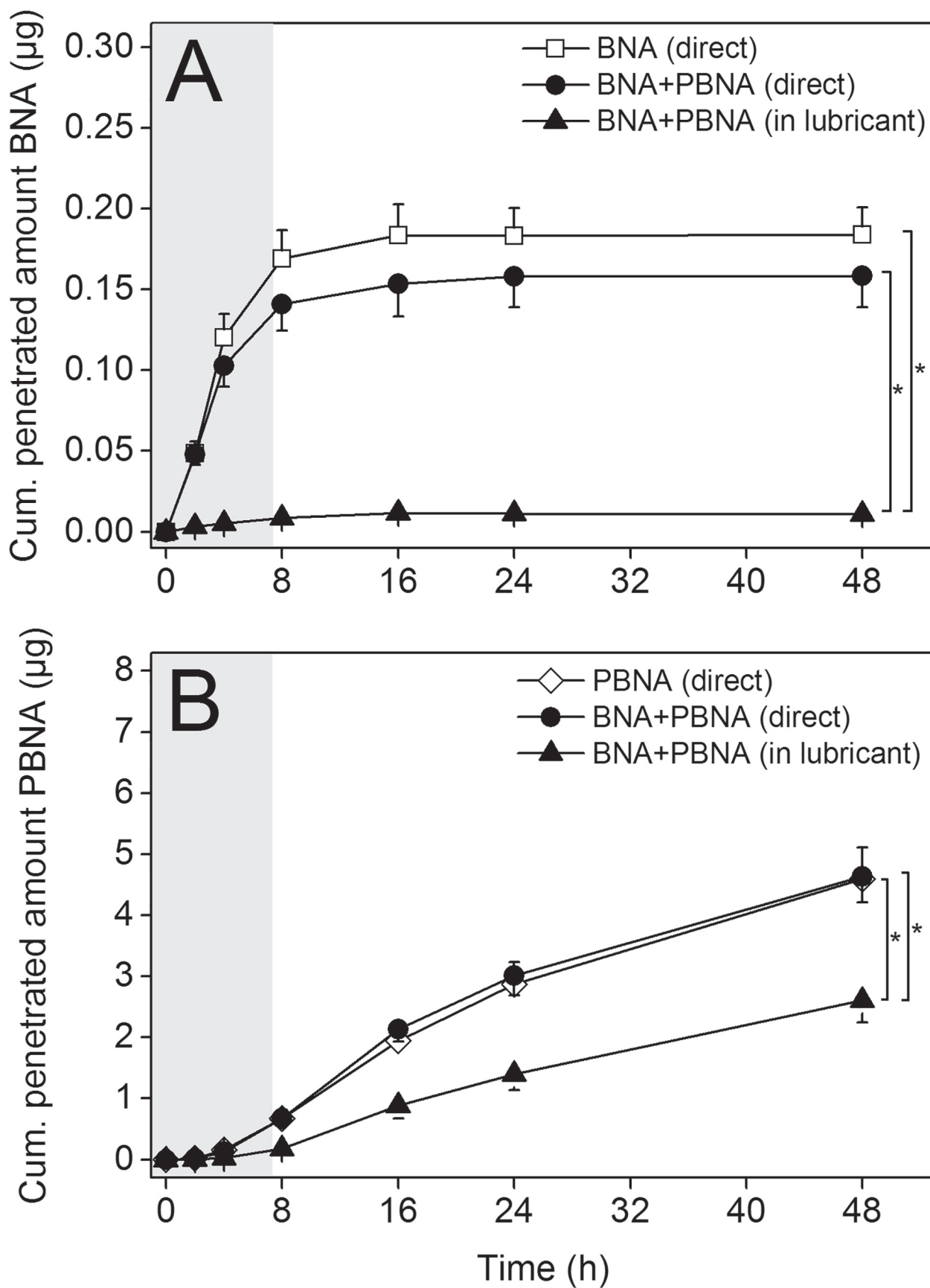


Fig. 1. Cumulative penetrated amounts (means \pm SEM) of (A) beta-naphthylamine (BNA; $n = 7$) and (B) *N*-phenyl-beta-naphthylamine (PBNA; $n = 7$) as a function of the exposure scenario. For both AA's, maximal cumulated amounts were shortened significantly by exposure of the aromatic amine in lubricant at 48 h ($p < 0.05$). The duration of exposure (8 h) is marked in grey.

Table 3
Dermal penetration data of BNA and PBNA through human skin.

Analyte	Exposure scenario	Amount of aromatic amines (μg ; means \pm SEM; $n = 7-8$)			Maximal flow rate ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Break-through time (h)
		Stratum corneum	Epidermis/Dermis	Receptor fluid		
BNA	BNA (direct)	0.006 \pm 0.001	0.005 \pm 0.001	0.184 \pm 0.017*	0.06 \pm 0.007*	0.63 \pm 0.14*
	BNA/PBNA (direct)	0.006 \pm 0.001	0.008 \pm 0.002*	0.159 \pm 0.020#	0.04 \pm 0.003#	0.18 \pm 0.12*
	BNA/PBNA (in lubricant)	n.d.	0.004 \pm 0.001*	0.011 \pm 0.001*#	0.002 \pm 0.003*#	0.22 \pm 0.23
PBNA	PBNA (direct)	7.03 \pm 0.62*	1.41 \pm 0.78*	4.60 \pm 0.51*	0.27 \pm 0.023*	3.93 \pm 0.46*
	BNA/PBNA (direct)	13.3 \pm 3.43#	6.03 \pm 2.21*	4.64 \pm 0.42#	0.25 \pm 0.021#	3.71 \pm 0.41#
	BNA/PBNA (in lubricant)	0.25 \pm 0.032*#	2.13 \pm 0.53	2.61 \pm 0.36*#	0.12 \pm 0.018*#	7.28 \pm 0.82*#

*;# Values in the same column and application subgroup sharing the same character are significantly different at $p < 0.05$.

BNA: beta-naphthylamine.

PBNA: *N*-phenyl-beta-naphthylamine.

n.d.: not detected.

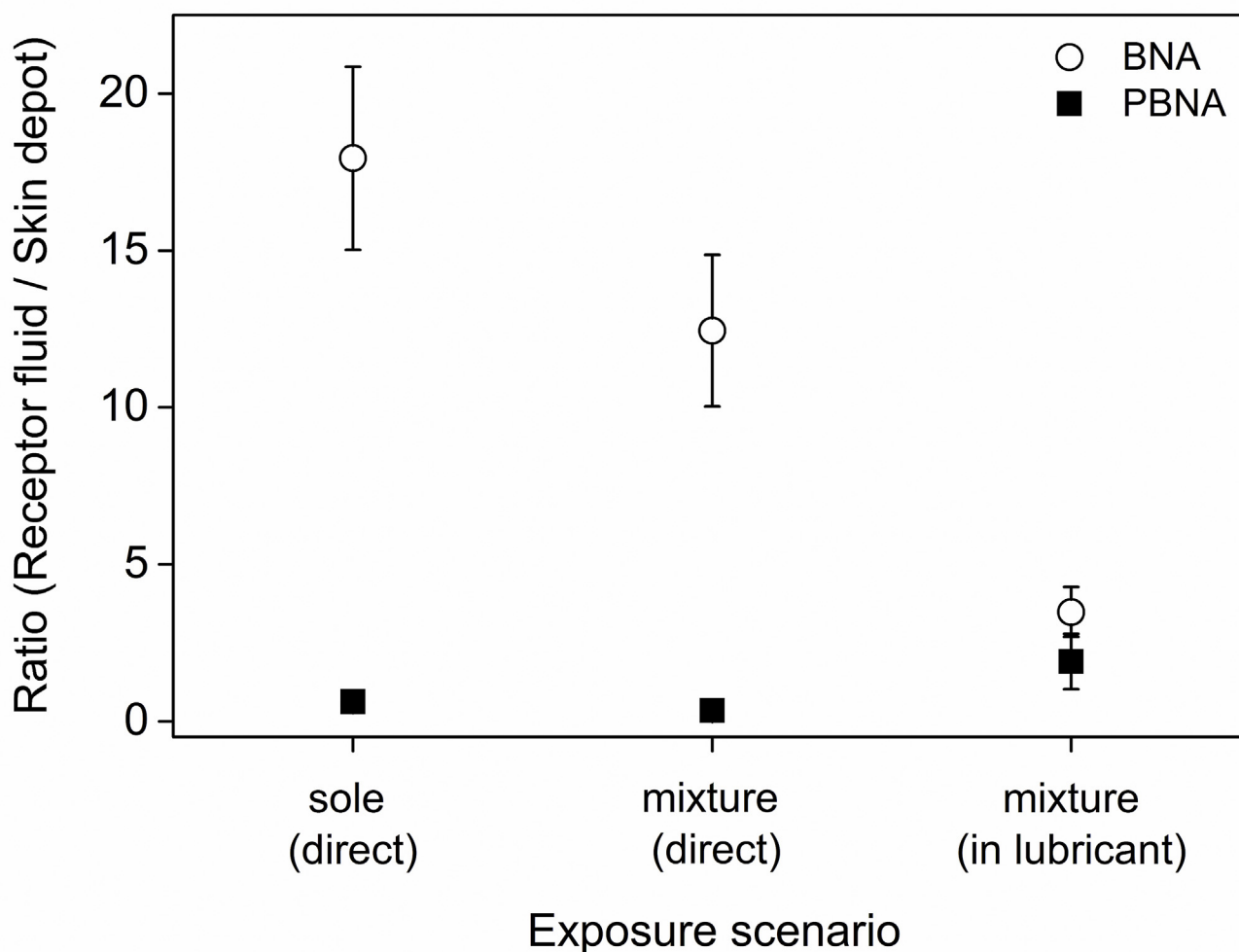


Fig. 2. Ratio of the amounts of beta-naphthylamine (BNA; $n = 7-8$) and *N*-phenyl-beta-naphthylamine (PBNA; $n = 7-8$) penetrating through the skin to the amount of the aromatic amines remaining in the skin for the different exposure scenarios at the end of experiments (48 h, means \pm SEM).

previous *ex vivo* study, where 54% of applied BNA was detected in the receptor fluid within 24 h of exposure of excised human skin (Lüersen et al., 2006). The discrepancies between the transdermal penetrated amounts can be explained by the 3-fold longer exposure period of 24 h in the study of Lüersen et al. (2006) compared to the current study. However, the amounts of BNA penetrating within the first 7 h of exposure were in the same range as in the present study after 8 h (29 vs. 33% of applied amount). This is surprising since Lüersen et al. (2006) performed the experiments under

occlusion, which can enhance the penetration of lipophilic compounds (Guy et al., 1987; Treffel et al., 1992). One reason might be that pure saline was used as receptor solution in the study of Lüersen et al. (2006). Thus, the penetration of the lipophilic AA from skin into the hydrophilic solution might be slowed down and/or delimited. The prolonged lag time of 1.2 h additionally supports the assumption of a delayed transfer. With $0.51 \mu\text{g cm}^{-2} \text{h}^{-1}$ Lüersen et al. (2006) determined a 9-fold higher flux compared to our results, which is reasonable considering the higher charging

with BNA applied in the experiments (9.6 vs. 0.8 $\mu\text{g}/\text{cm}^2$).

With ~2% of the applied dose shifting to the receptor fluid within 48 h, the present study demonstrated and quantified for the first time the penetration of PBNA through human skin. This is in contrast to a previous *ex vivo* study investigating the dermal penetration of several AA's through human skin. Even after 24 h of exposure Wellner et al. (2008) have not been able to detect PBNA in the receptor fluid of diffusion cells. A likely reason may be the addition of human albumin to receptor fluid in the present experiments, which closer resembles the *in vivo* physiological conditions and enhances the solubility of lipophilic substances. As PBNA is of low water solubility with a high LogP value the pure aqueous receptor fluid (saline) used by Wellner et al. (2008) probably impedes the diffusion of PBNA out of the skin into the receptor fluid.

So far, no data on dermal deposition of BNA and PBNA are available. For this purpose, the formation of a relevant skin reservoir, as described for other chemicals (Roberts et al., 2004; Schneider et al., 2016), should be considered, particularly in studies investigating the dermal penetration of hazardous substances. Depletion of the skin reservoir might exceed the actual exposure period by far and extend the overall systemic uptake. Although the intradermal amounts of BNA and PBNA at the end of experiments were similar (2.1 vs. 3.2% of applied dose), different migration patterns into the skin as well as from the skin into receptor phase were observed. BNA penetration into the receptor fluid ceased when it was removed from the skin (Fig. 1). With about 94% of absorbed BNA detected in the receptor fluid at the end of the experiments, the skin does not seem to serve as a reservoir for this amine. Furthermore, a constant penetration flow of BNA within the skin as well as between skin and receptor fluid can be assumed since BNA was evenly distributed in the stratum corneum and the deeper skin layers (Table 3). In contrast, the situation is quite different for PBNA. The intradermal distribution indicates that the skin acts as a sink for PBNA absorption and therefore as a barrier for further penetration. This assumption is supported by PBNA's long break-through times (up to 7.3 h) and low overall penetration rates (2–9% of applied dose in 48 h). The formation of a relevant skin reservoir can be explained by the lipophilic character of PBNA impeding its diffusion through skin, which results in dermal accumulation of the substance.

Occupational or environmental dermal contact to chemicals usually means exposure not only to a single agent but co-exposure to many chemicals simultaneously. It has been shown that different vehicles, e.g., organic solvents or surfactants can affect the penetration of a substance applied on skin (Skowronski et al., 1994; Park et al., 2000). However, the mutual influence of co-applied substances on their dermal penetration pattern has been studied only to a limited extent. An increase of transdermal flux (up to factor 5.2) and intradermal uptake (up to 6–fold) was reported by Romonchuk and Bunge (2010) following co-exposure to the cosmetic ingredients methyl paraben and 4-cyanophenol compared to single application. In the present study, co-application of both AA's increased the dermal amount of PBNA overall (factor 2.3; $p < 0.05$) as well as in relation to the transdermal penetrated amount (Fig. 2) which is in agreement with the results of Romonchuk and Bunge (2010). However, even with an increase in intradermal accumulation of PBNA, transdermal flux and amount of this amine in the receptor fluid was similar for both application modes, i.e. mixed or sole application indicating that the spatial distribution of PBNA within the different layers of the skin is altered by co-application. This could have several reasons, based mainly on the principles of diffusion. One reason could be that the uptake of PBNA in the receptor fluid is restricted due to solubility reasons; however, in the present study the penetrated amounts of PBNA measured in the receptor fluid are well within the range of solubility. This might be different within the several skin layers as well as at the interfaces

between these layers or between the skin and the receptor fluid. The traverse from skin into the receptor solution includes a passage through an aqueous boundary layer which can become rate-limiting for the diffusion of substances (Diez-Sales et al., 1991). In experimental studies (Stehle and Higuchi, 1972) it was shown that even by vigorously stirring of the receptor fluid, this physical barrier cannot be ultimately removed. In addition, highly hydrophobic substances undergo viable tissue controlled diffusion, thus, the high water content in the epidermis (~70% of its mass) probably limits the passage of PBNA through this layer and into the receptor fluid. Though for BNA the intradermal uptake was also increased (factor 1.3), the transdermal penetrated amount was slightly decreased when co-applied with PBNA (Fig. 2). As the combined amounts of BNA in skin and receptor fluid are basically the same with both exposure scenarios the traverse through skin seems to be slowed in the presence of PBNA, leading to a reduced flux in conjunction with an increased intradermal amount of BNA even 40 h after exposure.

Certain oily or fatty topical vehicles might have an influence on the penetration process especially of lipophilic compounds by reducing the barrier resistance of the stratum corneum and therefore facilitating the penetration of the substance into the stratum corneum lipids. The saponification process during manufacture of the industrial lubricant generates free fatty acids. For fatty acids an interaction with the hydrocarbon chains of the bilayer lipids is assumed (Trommer and Neubert, 2006). Thereby the lipid packing order within the bilayers of the stratum corneum is disturbed facilitating the penetration of lipophilic substances like PBNA. This facilitating effect on the penetration of o-toluidine through human skin applied in a polymer lubricant was reported by Korinth et al. (2008). However, both, the intradermal uptake and the transdermal penetration of BNA and PBNA were diminished when exposed in lubricant compared to neat application (Table 3). The structure of industrially produced lubricant resembles a sponge based on crystallized fatty acid calcium salts. Within this crystalline structure, base oils and other ingredients like the AA's are kept by a combination of Van der Waals and capillary forces (Westerberg et al., 2015) limiting its movement within the grease and therefore restricting the supply into the skin. The deceleration of the percutaneous penetration by the limited supply from parts of the donor material not touching the skin is also reported in studies using soil as donor material (Beriro et al., 2016). The differences between the penetrated amounts for BNA and PBNA for neat exposure compared to exposure in lubricant (factor ~19 vs. ~6) could have several reasons. On the one hand, BNA might be anchored more tightly in the structure of the lubricant than PBNA leading to a fast depletion of superficial BNA. On the other hand the overall difference in applied concentration of the amines, with PBNA ~500 times higher concentrated than BNA, could have led to the faster depletion of superficial BNA. The fact that the amount of BNA in the stratum corneum was below the detection limit is probably caused by the fast penetration of the amine. Since only small amounts of PBNA penetrated the skin with high break through times, depletion might not be a relevant factor for this amine. In addition, by the absorption of only small PBNA amounts saturation within the skin or solubility problems might not occur, explaining that no intradermal accumulation of PBNA was observed when applied in lubricant compared to neat exposure (Table 3; Fig. 2).

5. Conclusions

The *ex vivo* diffusion cell model used in the present study has been proven to be an appropriate approach for investigating the dermal penetration the aromatic amines BNA and PBNA under an occupational exposure scenario. The results verify the percutaneous

penetration of BNA and, for the first time, prove the transdermal penetration of PBNA through human skin. It can be concluded from the present study that human skin serves as a relevant barrier for the systemic uptake of PBNA, but not of BNA. Furthermore, the study clearly reveals that dermal penetration data obtained by neat exposure to BNA and PBNA might not be valid for other exposure scenarios since dermal uptake of the AA's varies with co-exposure and formulation.

Conflict of interest

The authors declare that they have no conflict of interest.

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