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Estimation of human percutaneous bioavailability for two novel brominated flame retardants, 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB) and bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP)

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Abstract

2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) and bis(2-ethylhexyl)tetrabromophthalate (BEH-TEBP) are novel brominated flame retardants used in consumer products. A parallelogram approach was used to predict human dermal absorption and flux for EH-TBB and BEH-TEBP. [^{14}C]-EH-TBB or [^{14}C]-BEH-TEBP was applied to human or rat skin at 100 nmol/cm^2 using a flow-through system. Intact rats received analogous dermal doses. Treated skin was washed and tape-stripped to remove "unabsorbed" [^{14}C]-radioactivity after continuous exposure (24h). "Absorbed" was quantified using dermally retained [^{14}C]-radioactivity; "penetrated" was calculated based on [^{14}C]-radioactivity in media (*in vitro*) or excreta+tissues (*in vivo*). Human skin absorbed EH-TBB (24±1%) while 0.2±0.1% penetrated skin. Rat skin absorbed more (51±10%) and was more permeable (2±0.5%) to EH-TBB *in vitro*; maximal EH-TBB flux was 11±7 and 102±24 pmol-eq/cm^2/h for human and rat skin, respectively. *In vivo*, 27±5% was absorbed and 13% reached systemic circulation after 24 h (maximum flux was 464±65 pmol-eq/cm^2/h). BEH-TEBP *in vitro* penetrance was minimal (<0.01%) for rat or human skin. BEH-TEBP absorption was 12±11% for human skin and 41±3% for rat skin. *In vivo*, total absorption was 27±9%; 1.2% reached systemic circulation. *In vitro* maximal BEH-TEBP flux was 0.3±0.2 and 1±0.3 pmol-eq/cm^2/h for human and rat skin; *in vivo* maximum flux for rat skin was 16±7

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Chemical compounds studied in this article

2-ethylhexyl 2,3,4,5-tetrabromobenzoate (PubChem CID: 71316600; CAS No. 183658-27-7, FW: 549.92 g/mol, logP_{est}: 7.73-8.75 (1, 2)). Other published abbreviations for 2-ethylhexyl-2,3,4,5-tetrabromobenzoate are TBB, EHTeBB, or EHTBB.

bis(2-ethylhexyl) tetrabromophthalate (PubChem CID: 117291; CAS No. 26040-51-7, FW: 706.14 g/mol, logP_{est}: 9.48-11.95 (1, 2)). Other published abbreviations for bis(2-ethylhexyl)tetrabromophthalate are TeBrDEPH, TBPH, or BEHTBP.

pmol-eq/cm^2/h. EH-TBB was metabolized in rat and human skin to tetrabromobenzoic acid. BEH-TEBP-derived [^{14}C]-radioactivity in the perfusion media could not be characterized. Less than 1% of the dose of EH-TBB and BEH-TEHP is estimated to reach the systemic circulation following human dermal exposure under the conditions tested.

Keywords

dermal bioavailability; brominated flame retardant; 2-ethylhexyl 2-3,4,5-tetrabromobenzoate; bis(2-ethylhexyl) tetrabromophthalate; parallelogram method; persistent organic pollutant

Introduction

Flame retardant (FR) chemicals are added to consumer products and building materials to decrease the risk of fire (3). However, FRs are also environmental pollutants, especially when incorporated into products as additive agents (4-7). After decades of consumer use it was concluded that pentabrominated diphenyl ether mixtures (pentaBDE), primarily used as FRs in polyurethane foams, bioaccumulate and have undesirable toxicity profiles with evidence for thyroid, liver, neurological, and reproductive toxicities, and cancer endpoints (7-14). As such, pentaBDE (and octaBDE) formulations were voluntarily withdrawn from the US marketplace by their manufacturers at the end of 2004 while decaBDE formulations were withdrawn in 2013 (15). This restriction on the use of pentaBDE has resulted in the utilization of novel brominated FRs as replacements. Penta- and octaBDE congeners are included under the United Nations Environmental Programme (UNEP) Persistent Organic Pollutants (POPs) list (16). As a result, polyurethane foam for soft furnishings produced after 2004 contains a mixture of brominated and chlorinated FRs, including tris(1,3-dichloro-2-propyl) phosphate (TDCPP; “chlorinated tris”), 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB), and bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP), among others (3, 17). EH-TBB and BEH-TEBP are used in couch foam and baby products (mattresses and high-chair foam). In addition, BEH-TEBP is used as a FR or plasticizer in polyurethane foams, flexible polyvinyl chloride, adhesives, carpet backing, fabric coating, film and sheeting, wire and cable insulation, and wall coverings while the only known application for EH-TBB is in polyurethane foam.

EH-TBB and BEH-TEBP have been found in dust collected in the US, Europe, Oceania, and Asia, indicative of the global distribution of FR foams in consumer products (18-24). In addition to household and office dust, EH-TBB and BEH-TEBP are found worldwide in outdoor dust, sediment, and wildlife (3, 4, 18, 19, 25-28). In studies of the Great Lakes atmosphere, both chemicals appear to be increasing with calculated doubling times of 3-6 years (29). Both EH-TBB and BEH-TEBP are slated to undergo a full risk assessment under the Toxic Substances Control Act (TSCA) Work Plan and Action Plan (30). US national production volume for BEH-TEBP in 2012 was 1,000,000 - 10,000,000 lb/yr. Neither EH-TBB production and import volumes to the US, nor international production volumes are publically available (31). However, EH-TBB is not listed in the US EPA High Production Volume Information System, indicating its US production and import volumes are less than the threshold of “1 million pounds or more per year”. Exact global production volumes for

EH-TBB and BEH-TEBP are unavailable; conservative estimates for total novel BFR production is 100,000 tons/year (32, 33). Both EH-TBB and BEH-TEBP have low vapor pressures, high lipophilicity (estimated log P of 7.73-8.75 and 9.48-11.95, respectively (1, 2)), as well as high persistence and bioaccumulation characteristics (19, 29). Toxicity profiles for both chemicals are poorly described (33, 34).

Several studies have detected EH-TBB, BEH-TEBP, or their metabolites in human samples (35, 36). Precise routes of exposure are unclear but ingestion and inhalation of FRs in dust has been well documented (37-40). In addition, dermal contact with FRs has been associated with systemic exposures (4). Unfortunately, few studies have investigated the role of dermal uptake despite repeated demonstration of strong positive correlations between FR levels in the indoor environment (e.g., dust), on human skin (hand wipe collections), and in the bodies of adults and children (serum concentrations) (4, 27, 41). Dermal bioavailability of legacy brominated flame retardants (i.e., BDEs) in humans has been investigated (42-44) but very little is known about the dermal disposition of novel brominated flame retardants (44).

Previous disposition studies investigating EH-TBB and/or BEH-TEBP alone or in commercial preparations (Firemaster 550, Firemaster BZ-54, Uniplex FRP-45), in mammals (45-47) or fish (48), found EH-TBB was more readily absorbed from the gut and excreted as metabolite(s) while BEH-TEBP was less likely to be absorbed but was more likely to bioaccumulate in liver and other organs after repeated administration. Disposition of newer formulations that contain EH-TBB and BEH-TEBP (e.g., Firemaster 600 (49)) have not been tested.

Here, *in vivo* studies were conducted using female Sprague Dawley (SD) rats and *in vitro* studies were conducted using split-thickness skin (i.e.., epidermis and upper portion of the dermis) from human donors and female SD rats exposed to 100 nmol/cm^2 radiolabeled EH-TBB or BEH-TEBP. This dose was selected based on expert opinion (50) and the need to apply enough [^{14}C]-radioactivity to detect the chemicals in the receptor fluid or excreta. Following 24 h exposure, the treated skin was washed and tape stripped. For these studies, the term ‘absorbed’ is used to describe the portion of the applied dose found within the skin and tape strips. Tape stripping may not be sufficient to completely remove the human stratum corneum (51), but to provide a conservative estimate for potential bioavailability, chemical recovered in tape strips was included in the ‘absorbed’ fractions calculations. Similarly, although dose retained within skin (‘absorbed’) may ultimately be removed by normal desquamation and never reach the bloodstream, amounts recovered in the ‘absorbed’ fraction were included in the estimations of bioavailability in an effort to provide conservative estimates for uptake. In descriptions of *in vitro* experiments, ‘penetrated’ is used to describe chemical that has completely diffused through the skin into the underlying fluid (termed ‘receptor fluid or perfusion media’), analogous to the amount reaching systemic circulation following *in vivo* exposure (52, 53). The sum of excreted and retained [^{14}C]-radioactivity in tissues outside the dosed skin was used to determine the total penetrated fraction *in vivo*. The values for penetration were used to estimate bioavailability and flux for EH-TBB and BEH-TEBP. Finally, the sum of ‘absorbed’ and ‘penetrated’ and the absorptive flux calculated for each model.

Methods & Materials

Chemicals

[^{14}C]-labeled EH-TBB and BEH-TEBP were custom synthesized by Moravek Biochemicals (Brea, CA) with the carboxyl carbon radiolabeled (Figure 1). [^{14}C]-EH-TBB (Lot # 256-063-055-A-20130423-DJI) had a radiochemical purity of 99.4% (specific activity = 55 mCi/mmol). [^{14}C]-BEH-TEBP (Lot # 256-061-0605-A-20130419-DJI) had a radiochemical purity of 99.9% (specific activity = 60.5 mCi/mmol). Radiochemical purity was confirmed by radio-HPLC using the methods described below (Figure 3(A) and Figure 4(A), respectively). Both chemicals had a chemical purity of >99%, as compared to their respective reference standard (Accustandard, New Haven, CT). 2,3,4,5-tetrabromobenzoic acid (TBBA; >98% pure) was purchased from the Duke University Small Molecule Synthesis Facility (Durham, NC). Scintillation cocktails were obtained from MP Biomedicals (Ecolume; Santa Ana, CA), Perkin-Elmer (Ultima Gold & PermaFluor E+; Torrance, CA), or Lablogic Inc., (Flow Logic U; Brandon, FL). All other reagents used in these studies were high performance liquid chromatography (HPLC) or analytical grade. Chemical structures were drawn using ACD/Labs Chemsketch (Advanced Chemistry Development, Inc., Toronto, Canada).

Flux calculation

Maximal flux (J_{ss}) was calculated for both *in vitro* and *in vivo* studies using the method described by Hughes et al. (54) and derived from Fick's first law of diffusion (55). Mass was calculated from the amounts of chemical recovered in media (*in vitro*) or in excreta (*in vivo*). Briefly, the maximal flux (pmol-eq per square centimeter per hour) was derived from the slopes of the penetrated mass across each barrier plotted versus sampling time period (Equation 1). The experimental duration was expected to be insufficient to produce significant depletion of the applied chemical, i.e., flux was not dose-limited.

Parallelogram calculation

The principles of the parallelogram approach to the dermal exposure assessments were used to estimate bioavailability following *in vivo* human systemic exposures to a relevant dose of dermally-applied chemical (Equation 2). Because of inconsistent differences in percutaneous absorption between rat and human skin, it is not possible to derive a general adjustment factor for estimation of human percutaneous absorption. However, when these data are available for rat *in vivo* and for rat and human skin *in vitro*, the *in vivo* dermal absorption through human skin can be estimated from the relationship outlined by the parallelogram method (56-59). Briefly, *in vivo* human exposure is estimated as a function of *in vitro* human exposure multiplied by a normalization factor based on the same dose applied to rat skin *in vivo* and *in vitro*. The parallelogram approach is based on the assumption that the ratio of *in vivo* to *in vitro* dermal penetrance of a chemical through the animal model's skin (here, female SD rat) is the same as the ratio of *in vivo* to *in vitro* dermal penetrance in humans.

In vitro experiments

In vitro studies were conducted as described previously by Knudsen et al (53). Briefly, full-thickness human skin was obtained from the National Disease Research Interchange (Philadelphia, PA, USA) from 4 Caucasian individuals aged 78-87 years old (2 male and 2 female, dorsal/scapular skin, excised ≤ 12 h post-mortem, shipped at −80 °C). Two of the human skin samples were of sufficient size to be sampled in both studies; skin from three separate individuals was used in studies for each individual chemical. The skin was shipped and stored frozen (−80°C) until use. Full-thickness female SD rat skin (N=4/chemical, 10-11 weeks old) was obtained from Harlan Bioproducts for Science (Indianapolis, IN, USA). Twenty-four hours prior to excision, hair on the dorsal surface was clipped; the day of shipment, the rats were humanely euthanized by CO_2 inhalation and skin excised. The skin was shipped on dry ice and stored at −80°C until use. *In vitro* dermal absorption tests were conducted according to the OECD Test Guideline 428 (60).

A flow-through diffusion cell system (0.64 cm^2 diffusional area; Crown Bio Scientific, Inc., Somerville, NJ, USA) using methodology as described by Bronaugh and Maibach (61) and Bronaugh and Stewart (62) was employed. Experiments for each species/chemical combination were run on separate days. On the day of the experiment, human or rat skin was thawed, direction of hair growth assessed and dermatomed to approximately 300 μm thicknesses using a Padgett dermatome (Kansas City, MO, USA) before placement in receptor fluid. Average sample thickness is shown in Table 1. The integrity of each human skin sample was tested using tritiated water. Samples with scintillation cocktail alone were used as background and the instrument was calibrated with [^{3}H] and [^{14}C] standards prior to each use. Penetrance of <0.05% of applied [^{3}H]-radioactivity was indicative of an intact barrier, analogous to healthy skin and samples that passed this test were used in these studies.

[^{14}C]-EH-TBB or [^{14}C]-BEH-TEBP in toluene (100 nmol/cm^2, ~1 μCi, 5 uL dose volume) were applied using a blunt tip Hamilton syringe (Franklin, MA, USA) to human and rat skin discs. The specific activity precluded testing at lower doses. Toluene was used as a vehicle due to limited solubility of the test chemicals; both EH-TBB and BEH-TEBP were observed to have limited solubility in more traditional solvents such as ethanol or acetone. The small dose volume combined with the rapid volatilization of the solvent (the flow-through cells were open to the air and the whole system was placed a fume hood) minimized physiological effects (e.g., skin delipidation by toluene). After 24 h, the epidermal surface (with the cell top in place to recover chemical on the surface of the skin sample) was washed six times with 0.5 ml of a mixture of Joy® liquid soap:water (1:1) using a 1 mL pipette to remove unabsorbed chemical. The skin wash fractions were pooled into two vials and mixed with scintillation cocktail. The cell top and cell body were individually washed three times with 0.5 ml ethanol. Skin samples were dried overnight in a hood. The following day, each skin disk was tape stripped up to 10 times with clear tape to remove the stratum corneum; when the stratum corneum visibly separated, tape stripping ceased. Tape stripping was anticipated to remove much of the stratum corneum. Although the stratum corneum is a minimally viable layer that is continuously lost, to be risk conservative, dose fractions recovered in tape strips were assumed to represent absorbed chemical after 24 h. Washed

and stripped skin was then chemically solubilized in 1 ml of Soluene 350 (Perkin Elmer) overnight in a water bath set at 37°C. Hionic Fluor (Perkin Elmer) was added to the dissolved skin solution, and "absorbed" [^{14}C]-radioactivity was quantified.

In vivo experiments

In order to link data characterizing the fate of orally-administered EH-TBB (46) and BEH-TEBP (63) in the female rat and the *in vitro* skin studies described above, 100 nmol/cm^2 was applied to the dorsal surface of female SD rats (N = 4 rats/chemical, 11 weeks old, approximately 200 g, Harlan Laboratories, Indianapolis, IA). One day prior to dosing, animals were lightly anesthetized by isoflurane inhalation and an electric clipper was used to remove hair from the dorsal scapular region. Clipped areas were visually inspected for any nicks or cuts; animals were not used if nicks or cuts were found. Animals were returned to polycarbonate shoebox cages for recovery from anesthesia. Immediately prior to dosing, animals were again lightly anaesthetized by isoflurane inhalation, the dosing area visually inspected for nicks and a 1 cm^2 area marked. Dosing solution (as described for *in vitro* samples) was applied inside the marked area using a 25 μL negative displacement pipette with a flexible tip. The vehicle was dried with gentle fanning and the dosing site was then covered with a non-occlusive steel mesh cap attached with polyacrylate glue to prevent ingestion of the test article. After dosing, animals were placed in plastic Nalgene metabolism cages for collection of feces & urine. Animals were provided rat feed (NIH 31; Ziegler, Gardners, PA, USA) and tap water for *ad libitum* consumption.

Feces, urine, and cage rinses were collected and analyzed as described previously by Knudsen et al. (64) at 4, 8, 12, and 24 h. Animals were euthanized by CO_2 inhalation after 24 h. Following euthanasia, blood was collected by cardiac puncture, treated skin was excised, and complete necropsies were performed as described previously (64). Skin from the application area was treated in accordance with the OECD 427 method for *in vivo* testing of chemicals (65). Briefly, the skin was swabbed 6 times using a 10% Joy® liquid soap solution, 3 times using distilled water, and air dried overnight. Dried skin was tape stripped until the stratum corneum was visibly removed (approximately 10 times) using clear tape. Feces were air-dried and ground to a powder using a mortar and pestle. Tissues (including dosed skin) and feces were sampled in triplicate (approximately 25 mg/sample), combusted in a Packard (Waltham, MA, USA) 307 Biological Sample Oxidizer, and [^{14}C]-radioactivity content was quantified by liquid scintillation counting (LSC) analysis. Skin swabs and tape strips were analyzed by direct LSC as were triplicate samples of urine and cage washes. Data of the combusted tissues and feces, urine, cage washes, skin swabs and tape strips were used to compute an arithmetic sum of residual [^{14}C]-radioactivity. Tape stripping may not be sufficient to completely remove the human stratum corneum (51), and to be risk conservative, radioactivity recovered in tape strips as well as compound in skin that was tape stripped was considered "absorbed". [^{14}C]-radioactivity in cage washes and urine were combined in the summary urinary recoveries.

Samples of air-dried feces (~500 mg) were extracted with 2 × 2 mL prechilled 1:1 methanol/distilled water. Supernatants from the methanol/water were pooled into glass vials, concentrated to near dryness under vacuum, and reconstituted in 500 μL of HPLC-grade

water. Remaining samples were sequentially extracted with 2 × 800 μL of pre-chilled 3:1 dichloromethane/methanol, 1 × 1-2 mL acetonitrile, 1 × 1-2 mL hexanes, and 2 × 2 mL toluene. Organic supernatants were pooled into glass vials, concentrated to near dryness, reconstituted in 500-700 μL of 1:1 ethyl acetate/ethanol, and injected onto the HPLC. Samples of each supernatant (10-100 μL) were subjected to LSC to determine if further extraction was necessary; when samples contained less than 3 × background counts, extractions were deemed exhaustive. Remaining sample pellets were air dried, weighed, and un-extracted [^{14}C]-radioactivity remaining in the sample was quantified by combustion in a Packard 307 Biological Sample Oxidizer followed by LSC counting. Average extraction efficiency for BEH-TEBP study feces was 90% while extraction efficiencies for EH-TBB study feces was ~50%, based on [^{14}C]-radioactivity remaining in the sample.

The protocol for chemical extraction from dosed skin was adapted from Want et al. (66). Prior to extraction, skin was washed and tape stripped as described above. Briefly, samples of dosed skin (23 - 200 mg) were transferred to microcentrifuge tubes and soaked in 1 mL of 1:1 methanol/ distilled water at 4°C. Samples were further minced then transferred to bead-beater tubes. Homogenization occurred in a FastPrep-24 5G benchtop homogenizer (MP Biomedicals) at 6500 Hz for two cycles of 40 seconds with a 1 minute pause. Aqueous supernatants were transferred to microcentrifuge tubes. Remaining samples were sequentially extracted with 1.6 mL of prechilled 3:1 dichloromethane/methanol, 1 mL acetonitrile, 1 mL of hexanes, and 1 mL toluene with the above homogenization settings. Organic supernatants were pooled into glass vials, concentrated to near dryness (Savant SPD1010 SpeedVac), reconstituted in 500 μL of acetonitrile, and injected onto the HPLC. Average extraction efficiency was 96%, based on remaining [^{14}C]-radioactivity in samples.

Urine samples (500 μL) were filtered through PVDF centrifugal filters (Merck Millipore), added to 100 μL of ethanol, and spun down via centrifuge. Supernatants were transferred to glass vials and injected onto the HPLC.

HPLC-radiochemical analyses

The HPLC system used for analysis of receptor fluid (from the *in vitro* studies) and extracts from dosed skin, urine, and feces (from the *in vivo* studies) was composed of an Agilent (Santa Clara, CA, USA) 1100 HPLC system with an in-line INUS β-RAM3 radiochemical detector. Mobile phases consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). EH-TBB study samples were separated using a Restek (State College, PA) Raptor biphenyl column (2.7 μm, 4.6 mm×50 mm). Elution involved a gradient method: initial conditions (99% A) were maintained for 1 min; A was then reduced to 0% over 1 second and held at 0% A for 5 min at a flow rate of 1 mL/min. BEH-TEBP study samples were separated using the same column and mobile phases with a gradient method: initial conditions (60% A) were maintained for 5 min; A was then reduced to 10% over 2 min then to 0% A over 13 min at a flow rate of 1 mL/min. In all cases, the column was returned to initial conditions and allowed to equilibrate for 2 min before re-use. Scintillant flow (Inflow ES, Lablogic Corp.) was maintained at 2 mL/min initially and increased to 4 mL/min around regions of interest. Laura4 (Lablogic Corp.) software was used for instrument control and analysis software.

Statistical analysis

The data were subjected to statistical analysis using two-way ANOVA followed by the Tukey-Kramer test for pairwise comparisons (GraphPad Prism 6, GraphPad Software, Inc., La Jolla CA). Values were considered to be significantly different at $p < 0.05$.

Results

EH-TBB studies

In vivo (rat) and *in vitro* (rat and human) studies were performed to determine the dermal uptake of a single dose of EH-TBB (~100 nmol/cm^2) over 24 h. EH-TBB was well absorbed into and penetrated through skin both *in vitro* and *in vivo. In vitro* data showed that penetration over time was significantly lower in human skin ($p<0.05$) after EH-TBB application (Figure 2). Receptor fluid from human samples dosed with EH-TBB contained 0.2% of the applied [^{14}C]-radioactivity whereas approximately 2% passed through the rat skin in 24 h. The fractional recoveries (expressed as percent of administered dose) for unabsorbed dose (washes), absorbed ([^{14}C]-radioactivity in tape strips and retained in the skin), and penetrated (*in vitro* receptor fluid or *in vivo* tissues and excreta) are shown in Table 2. When EH-TBB was administered to the dorsal surface of female SD rats, approximately 10% of the dose was recovered in the skin at the dosing site (absorbed) and 11% was present in tissues or excreta (penetrated). In this same group, 6% of the dose was recovered in urine while 1% of the dose was recovered in feces through 24 h while blood and other tissues contained 4-5% of the administered dose (Table 3, Table S1). As observed in the *in vitro* studies, most of the administered [^{14}C]-radioactivity was recovered unabsorbed from the *in vivo* dosing site within 24 h of administration (Table 2). Over the 24 hour exposure period, 18-24% of the EH-TBB dose crossed into the skin and systemic circulation in the rat. From these studies it was determined that maximal EH-TBB flux through rat skin *in vitro* was 102±24 pmol-eq/cm^2/h and occurred between 6 and 12 h post-application. Maximum flux for rat skin *in vivo* was 464±65 pmol-eq/cm^2/h and occurred between 12 and 24 h post dose. Maximal flux for human skin (*in vitro*) occurred between 18 and 24 h and was 11±7 pmol-eq/cm^2/h. HPLC-radiometric analyses of perfusate, extracts, and excreta from EH-TBB studies demonstrated metabolism of EH-TBB to TBBA (Figure 3). Metabolism of EH-TBB was shown to occur in rat skin in the *in vitro* system. Only TBBA was detected in the receptor fluid. EH-TBB applied dermally to rats was primarily excreted in the urine as TBBA; extractable [^{14}C]-radioactivity from feces resolved as a small peak that also co-eluted with TBBA. Similar results were seen for perfusate collected from human samples. Based on the parallelogram methodology, conservative estimates predict approximately 10±3% of EH-TBB may be absorbed into human skin *in vivo*, with 0.8±0.6% reaching systemic circulation after 24 h of continuous exposure, likely in the form of TBBA.

BEH-TEBP studies

Dermal penetration of BEH-TEBP was lower compared to EH-TBB, both *in vivo* and *in vitro* (Table 4, Table S2, Table S3). Levels of BEH-TEBP [^{14}C]-radioactivity in perfusion media were approximately 100-fold lower than seen for EH-TBB. Penetration was significantly lower in human skin ($p<0.05$) at all time points after BEH-TEBP dosing. The nature of the radioactivity in the perfusion media for either rats or humans could not be

determined due to limits of detection for the HPLC system. However, HPLC-radiometric analyses of extracts from dosed skin and feces of rats showed all extractable [^{14}C]-radioactivity was recovered as parent (Figure 4). From these studies it was determined that maximal BEH-TEBP flux through rat skin *in vitro* was 1±0.3 pmol-eq/cm^2/h and occurred between 0 and 6 h post-application. *In vivo*, maximum flux for rat skin was 16±7 pmol-eq/cm^2/h and occurred between 12 and 24 h post dose. Maximal flux for human skin (*in vitro*) was 0.3±0.2 pmol-eq/cm^2/h and was reached between 0 and 6 h. Based on the parallelogram methodology, conservative estimates predict approximately 7.8±7.6% of BEH-TEBP may be absorbed into human skin *in vivo*; about 0.8±0.4% of the parent chemical is expected to reach systemic circulation after 24 h of continuous exposure.

Discussion

The objective of this study was to measure the dermal absorption of EH-TBB and BEH-TEBP in rat and human skin to provide an assessment of bioavailable fractions of both chemicals following *in vivo* dermal exposures to humans. As expected, based on known physicochemical characteristics, rat skin was more permeable to EH-TBB and BEH-TEBP than human skin *in vitro*. Further, human skin absorbed and retained much less of either chemical than rat skin. In BEH-TEBP studies, [^{14}C]-radioactivity that was absorbed into the skin was recovered as parent BEH-TEBP, as was [^{14}C]-radioactivity recovered in rat feces, which leads to the conclusion that dermally applied BEH-TEBP likely follows similar pathways as those found previously for oral- or intravenous-administered BEH-TEBP in the rat (63). Analyses of urine and extracts of skin and feces showed EH-TBB is metabolized in skin to TBBA and excreted as metabolite(s) in feces and urine after dermal absorption in the rat, putatively due to carboxylesterase activity. In the current study (as seen in Table 3), and consistent with previous studies conducted by the oral route in rats (46), there was minimal retention of either chemical in tissues after administration. Similar to previous observations for highly brominated polybrominated diphenyl ethers (PBDEs) (42), the skin appeared to act as a lipophilic “trap” for BEH-TEBP. Good hygiene practices may aid in decreasing residence time on the skin, which in turn could limit bioavailability and systemic exposure.

Strong positive correlations exist between FR levels in dust collected from homes schools and businesses, on hand wipes, and serum concentrations in adults and children (4, 27, 41); despite this, very little is known about the dermal disposition of FRs (44). The stratum corneum, a biologically inactive layer of the epidermis, is often the primary barrier to dermal absorption. However, given the highly lipophilic nature of EH-TBB and BEH-TEBP, diffusion into the stratum corneum may be significant; in these cases the viable epidermis and upper layers of the dermis are likely to be the primary barrier. However, given the observations that most of the material was washed off 24 h after application, it is likely that the stratum corneum is still an effective barrier for percutaneous uptake of EH-TBB and BEH-TEBP. Passive diffusion through this layer is governed by the lipophilicity of the agent and varies inversely with molecular weight (the MW of both compounds is > 500 g/mol). Once past the stratum corneum, a chemical may be metabolized *in situ* or move by diffusion or facilitated transport through the epidermal stratum geminativum, spinosum, and granulosum and into the dermis where it may enter the systemic circulation through capillary beds.

Skin is a metabolically active organ, with phase I (oxidative metabolism), phase II (conjugative metabolism), and phase III (transport) processes occurring (67-69). Organic anion transporting polypeptide (OATP) proteins have been shown to facilitate increased systemic exposure of PBDEs and other lipophilic chemicals in intestine and liver (70-72). At least one OATP transporter (OATP2B1) has been described in human skin (73) and human-derived keratinocytes appear to express functional OATP transporters, with immune-reactive staining apparent in viable regions of the epidermis (68). However, the role of transporters in the dermal absorption of EH-TBB and BEH-TEBP is not known.

A limitation of the study is the large mass of each compound applied to the skin. This was due to the low specific activity of the compounds, and the need to assure there was a sufficient amount of radioactivity applied to be detected in the receptor fluid, excreta, or tissues. Other than potential occupational exposure, it is unlikely that one would be exposed to the mass of either chemical applied in this study. The dose level tested was in excess of that detected in environmental samples (27) but given the possibility that lower surface loads may actually result in higher uptake efficiency (74), fractional recoveries and subsequent predictions for human exposure may actually under-predict the real-world bioavailable fraction experienced by continuously exposed individuals. In addition, residential exposures to these chemicals would be in the form bound to dust. For absorption to occur if the dust contacts the skin, the chemical would have to partition from the dust to the skin. While absorption of neat BFR in this study was observed, dermal absorption of BFR bound to dust would most likely be lower (75).

Another limitation was the choice of vehicle. Toluene is not a common vehicle for dermal absorption studies, but was chosen because it could more easily dissolve the test compounds than other vehicles such as acetone. Being an organic solvent, the toluene could have damaged the skin, primarily by altering the lipids in the skin (76), but we are uncertain of this potential effect. Also, a fraction of the toluene may have penetrated the stratum corneum and could have enhanced overall absorption. However, given the small volume applied (5 uL) and the volatility of the toluene, the skin effects of toluene in this experiment were most likely minimal.

Chronic exposure to BFRs and structurally similar chemicals via the skin is a common occurrence but only a few studies have been conducted to describe their dermal disposition. An *in vivo* dermal study of 2,2',4,4'-tetrabromodiphenyl ether (BDE 47) using female C57BL6 mice showed less than 10% of a 1 mg/kg dose eliminated in urine and feces over 24 h where approximately 77% of the dose was systemically available (e.g., recovered at the dose site or within tissues & excreta) over 5 days (77). *In vitro* studies using BDE-47 applied to human or rat skin found similar levels of penetration as those reported for BEH-TEBP (43). An *in vitro* dermal assessment of decabromodiphenyl oxide (BDE 209) using mouse skin estimated 2% of a 60 nmol dose was recovered in skin and receptor fluid (78). *In vitro* assessments of a series of PBDEs found lower levels of bromination corresponded to increased dermal penetration while more highly brominated congeners were more likely to accumulate in skin (42). Previous studies in this laboratory (53) estimated approximately 6% of a 100 nmol/cm^2 dose of tetrabromobisphenol A would be dermally bioavailable to humans based on *in vitro* human data (4%) normalized to rat *in vitro* and *in vivo* data (13%

and 22%, respectively). A recent study describing dermal disposition of ten different BFRs (including EH-TBB and BEH-TEBP) in full thickness human skin showed similar results when the chemicals were applied in ethanol and allowed to perfuse for 72 h (79).

Dermal absorption in children, especially infants, is different than adults (80). In the very young (i.e., the first few days of life), the keratinocyte layer has not fully formed, which has a substantial impact on dermal absorption (81). However, even after this layer has formed, the child's skin remains quite different from the adult for the first year of life (82). In adulthood, the barrier function(s) of the skin remain intact and may even increase as the skin ages, when transepithelial water loss decreases and the stratum corneum thickens (83). The relatively thin stratum corneum and small corneocytes found in infant skin has been proposed to result in weaker stratum corneum barrier function compared to that of adults (84). While we were unable to test skin from young individuals, the bioavailability and flux rates for these compounds may be higher in younger individuals, especially pre-term infants and newborns exposed to these chemicals.

Systemic quantification of internal EH-TBB and BEH-TEBP levels after occupational, consumer, and environmental exposures to dust containing these and other FRs likely results from at least two routes, ingestion and dermal contact. To quantify the dermal contact component, we applied the principles of the parallelogram approach to the dermal exposure assessments for both chemicals to estimate a likely level following *in vivo* human systemic exposures to a relevant dose of dermally-applied FR, as described by Ross et al (59). The hypothesis behind the parallelogram approach has shown data from rat to be within ±3-fold of the values measured in human subjects; however, when studies have been conducted under analogous conditions as part of a matched protocol study, the uncertainty for the predicted value is much lower (85-87). In the present study, the possible fraction remaining at the dosing site may eventually reach the systemic circulation or become removed by desquamation since steady state may not have been achieved during the 24 h study. In addition, these studies were designed to provide a conservative estimate for dermal uptake of these novel BFRs. In addition, the exposure matrix has been shown to significantly affect the bioavailability of flame retardants (88). This study, using neat compound applied directly to skin likely provides an upper bound estimate of possible absorption from soil or dust, or data relevant only to dusts that are fully saturated, an unlikely scenario for environmentally-relevant concentrations. Flux rates derived from this type of dose configuration likely represent an upper bound for dermal penetrance of these chemical and relationships between tested conditions and real-world exposures to EH-TBB and BEH-TEBP in dust should be further investigated. Therefore, these findings represent conservative assumptions, but may be useful in assessing the contribution of dermal exposure to aggregate exposures to susceptible populations. While percutaneous penetration was generally low, both compounds accumulated in the skin to varying degrees and may reach systemic circulation over time even after surface washing and removal, either as parent or metabolite (89).

These data provided herein are expected to aid in risk assessment for dermal exposures to EH-TBB and BEH-TEBP. Over a 24 hour period, the amounts of administered EH-TBB or BEH-TEBP that penetrated the human and rat skin *in vitro* continually increased for all samples, indicating that duration of exposure increases the risk of toxicity. Human skin is

less permeable than rat skin for a variety of chemicals (56, 90, 91) due to differences in anatomical, physiological, and biochemical factors (61, 85). These differences necessitate the use of a normalizing mechanism to account for differences in dermal uptake between species. Despite species differences in uptake, it is clear that EH-TBB can be absorbed by the skin and dermal contact should be expected to be a relevant route of exposure to humans. We anticipate these data will be useful in estimating human exposure risk, especially pregnant women and small children who are exposed to higher levels of household dust (37). This is of particular importance because, coupled with their increased surface area to volume ratio and immature detoxification pathways (92), early-life exposure to endocrine disrupting chemicals like EH-TBB and BEH-TEBP enhances susceptibility to diseases like obesity and other chronic pathologies (93, 94). There is increasing evidence that the combination of EH-TBB and BEH-TEBP is becoming the predominant replacement for pentaBDE in children's products (95, 96), increasing the likelihood of exposure in homes with small children and adults of child-bearing age.

Equation 1. Estimation of percutaneous flux.

$$J=\frac{\Delta m}{\Delta t A}$$

Equation 2. Estimation of human *in vivo* systemic exposure relative to the ratio of animal to human absorption (penetrated + absorbed) of dermally applied chemicals.

$$\mathrm{Human}_{\mathrm{in\ vivo}}=(\mathrm{Rat}_{\mathrm{in\ vivo}}/\mathrm{Rat}_{\mathrm{in\ vitro}}) \quad \times \quad \mathrm{Human}_{\mathrm{in\ vitro}}$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights for review

1. Human skin maximal flux was 11±7 (EH-TBB) & 0.3±0.2 (BEH-TEBP) pmol-eq/cm^2/h.
2. Predicted systemic bioavailability was <1% for either chemical after 24h.
3. Skin retained EH-TBB & BEH-TEBP after 24 h dermal exposure.
4. EH-TBB was hydrolyzed to tetrabromobenzoic acid; BEH-TEBP was not metabolized.
5. Skin contact is an important route of human exposure to EH-TBB & BEH-TEBP.

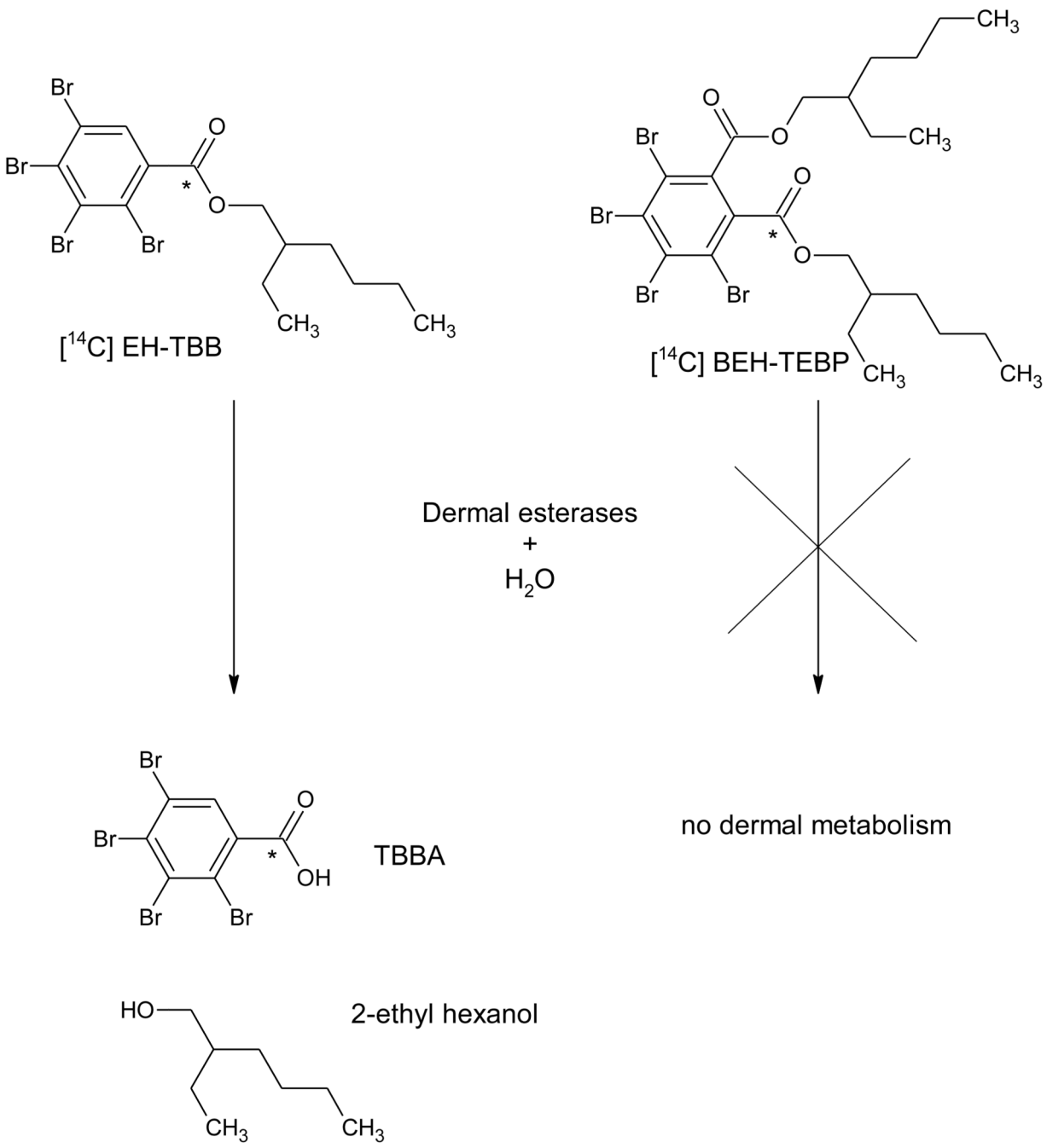
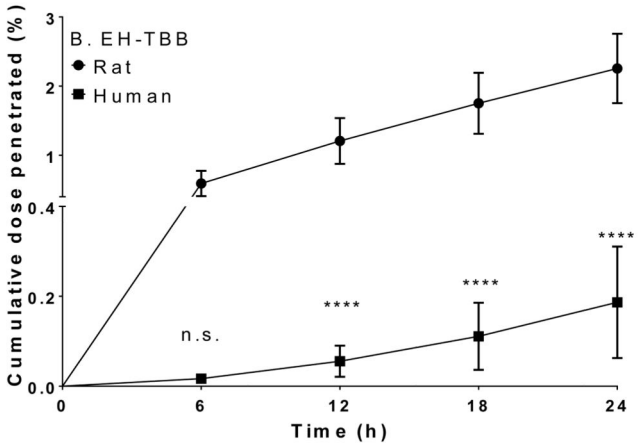


Figure 1.
Chemical structure and metabolism scheme for EH-TBB and BEH-TEBP; asterisk indicates the radiolabel location.



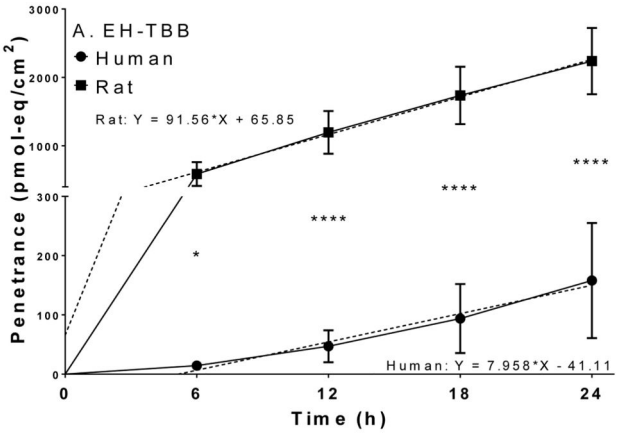


Figure 2.

Cumulative recoveries in the receptor fluid after a single application (100 nmol/cm^2) of [^{14}C]-EH-TBB to rat (●) or human (■) skin. A: Cumulative dose penetrated (%), B: Penetrance (pmol-eq/cm^2). Dashed lines show the linear regression of the penetrance data. Data represents mean ± S.D.; N=4 rat; N=3 human. *: p<0.05; **: p<0.01; ****: p<0.0001.

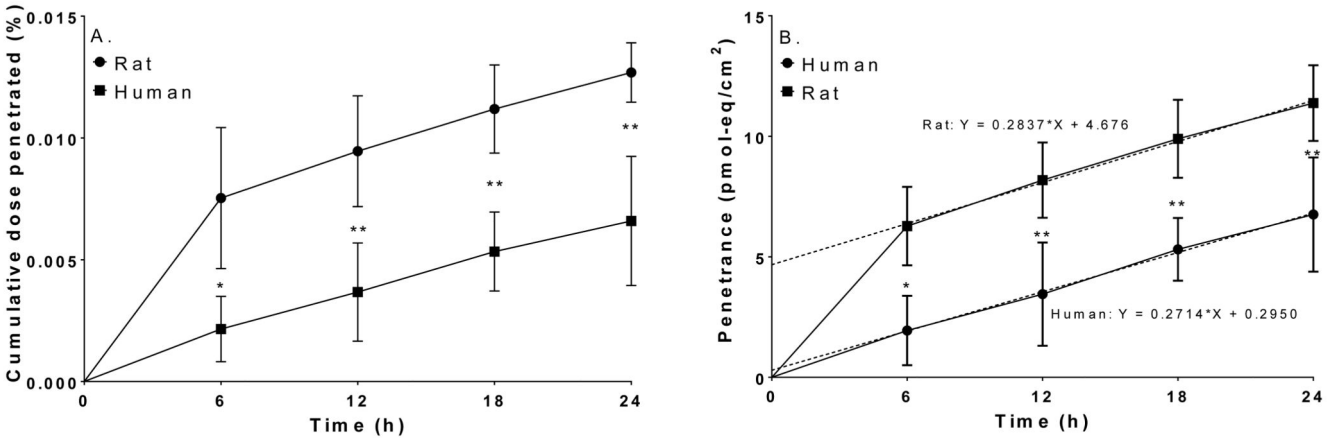


Figure 3.

Cumulative recoveries in the receptor fluid after a single application (100 nmol/cm^2) of [^{14}C]-BEH-TEBP to rat (●) or human (■) skin. A: Cumulative dose penetrated (%), B: Penetrance (pmol-eq/cm^2). Dashed lines show the linear regression of the penetrance data. Data represents mean ± S.D.; N=4 rat; N=3 human. *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$.

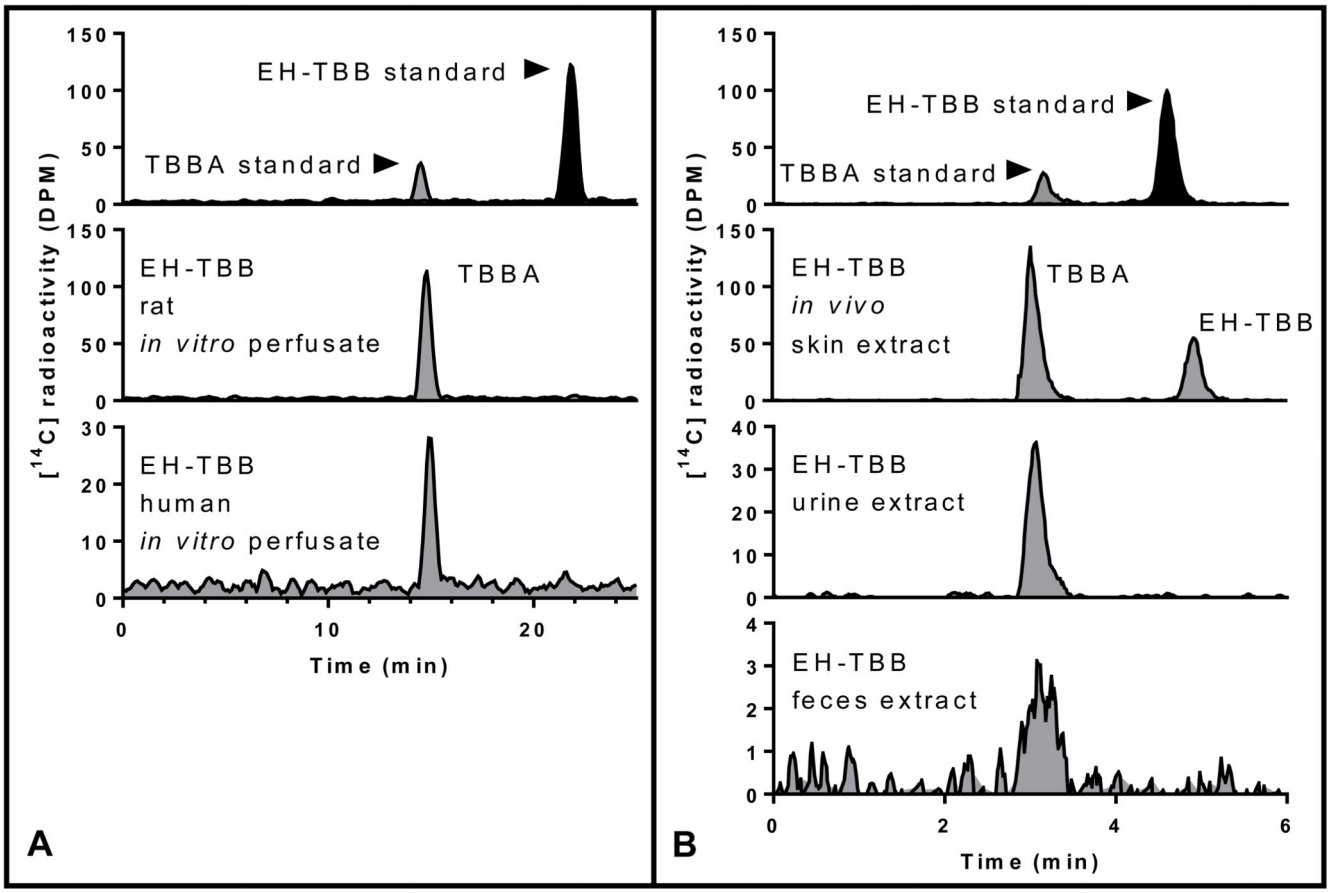


Figure 4.

Characterization of [^{14}C]-radioactivity in EH-TBB study samples from (A) *in vitro* media and B: extracts from *in vivo* samples (skin, urine, feces).

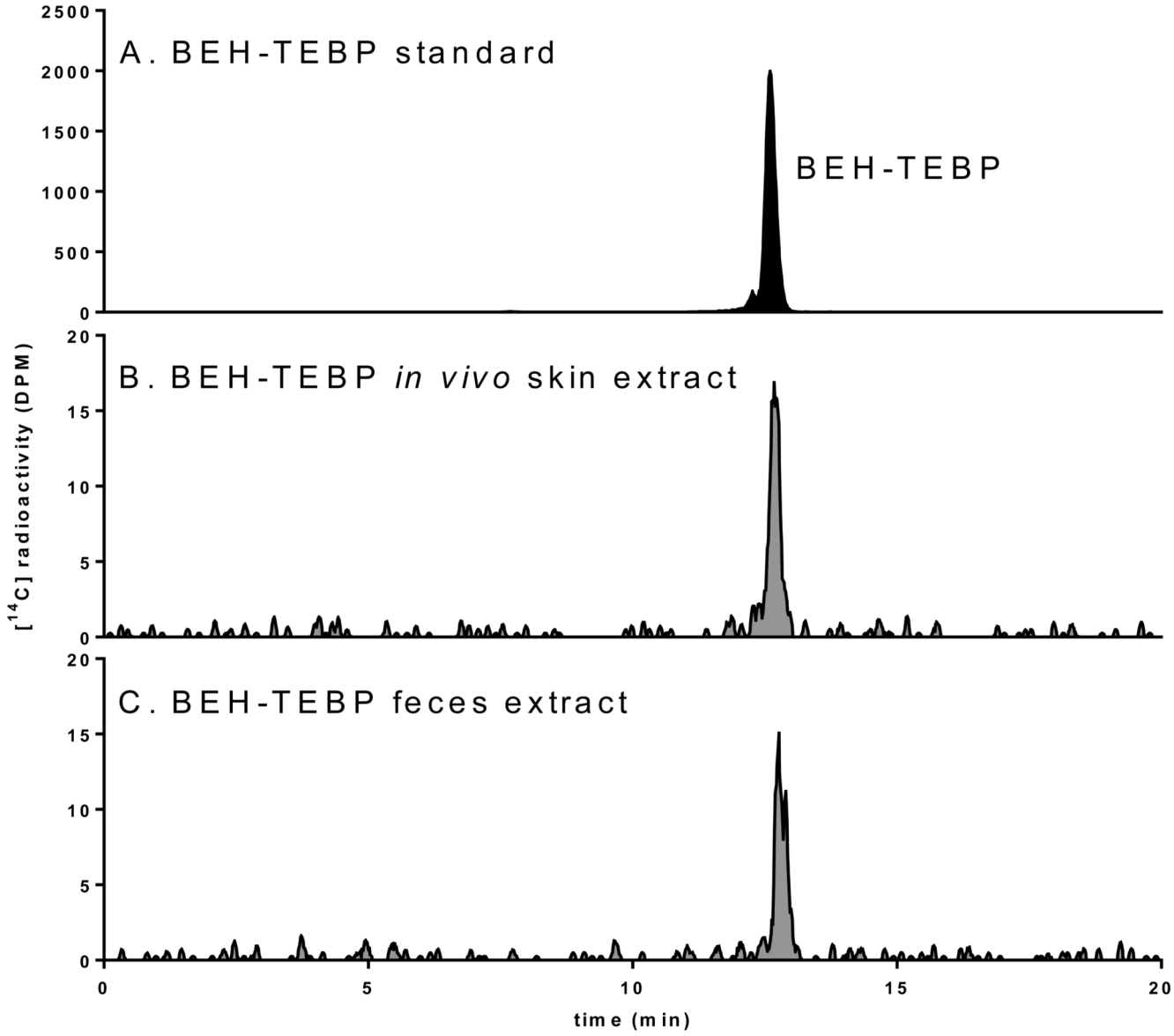


Figure 5.
Representative HPLC-radiochromatograms from BEH-TEBP studies: (A) BEH-TEBP standard, (B) *in vivo* rat study skin extracts, (C) *in vivo* rat study feces extracts.

Table 1

Skin sample thickness (μm) used in dermal studies, mean ± S.D.

EH-TBB		BEH-TEBP	
Rat	**Human**	**Rat**	**Human**
335 ± 50	414 ± 40	321 ± 32	433 ± 27

Table 2

EH-TBB studies: [^{14}C]-radioactivity recovery in various fractions at 24 h post-dose.

		EH-TBB		
Species		**Human (*in vitro*)**	**Rat (*in vitro*)**	**Rat (*in vivo*)**
Unabsorbed (%)	Washes	60 ± 9	37 ± 9	50 ± 5
	Cell	10 ± 4	4 ± 1	9 ± 2
Absorbed (%)	Tape strips	13 ± 4	17 ± 7	17 ± 4
	Skin	11 ± 5	34 ± 5	10 ± 3
Penetrated (%)		0.2 ± 0.1	2 ± 0.5	13 ± 1
Recovery (%)		95 ± 7	94 ± 7	98 ± 1

Table 3

[^{14}C]-radioactivity recovery in excreta & tissues following *in vivo* application of EH-TBB or BEH-TEBP (%).

	Feces	Urine	Blood	Non-GI tissues	GI tract	GI contents
EH-TBB	1 ± 0.3	6 ± 0.7	1 ± 0.4	3 ± 0.2	0.2 ± 0.02	1 ± 0.1
BEH-TEBP	0.3 ± 0.1	0.1 ± 0.03	0.3 ± 0.3	0.4 ± 0.3	0.01 ± 0.003	0.1 ± 0.1

Table 4

BEH-TEBP studies: [^{14}C]-radioactivity recovery in various fractions at 24 h.

	BEH-TEBP			
Species		**Human (*in vitro*)**	**Rat (*in vitro*)**	**Rat (*in vivo*)**
Unabsorbed (%)	Washes	80 ± 20	56 ± 6	63± 9
	Cell	4 ± 2	2 ± 0.4	6 ± 2
Absorbed (%)	Tape strips	4 ± 3	13 ± 5	19 ± 6
	Skin	8 ± 8	29 ± 2	8 ± 3
Penetrated (%)		0.007 ± 0.002	0.01 ± 0.002	1.2 ± 0.4
Recovery (%)		95 ± 7	98 ± 6	98 ± 2