Miljøministeriet
Miljøstyrelsen

January 2013.

Please note that this file is replacing the earlier draft version that by error was uploaded. The new file contains changes compared to the former file in the margin of safety paragraph page 25.

Update on uptake, distribution, metabolism and excretion (ADME) and endocrine disrupting activity of parabens 2009.

Julie Boberg, Camilla Taxvig, Sofie Christiansen and Ulla Hass,
Department of Toxicology and Risk Assessment,
National Food Institute, DTU.

Content

1 Summary

Parabens are preservatives used in a wide range of cosmetic products, including products for children. Additionally, methyl- and ethylparaben are permitted in certain foods in limited amounts. However, there is concern for endocrine disrupting effects at high exposure levels. This report summarizes conclusions of recent review papers and provides an overview of studies on uptake and metabolism as well toxicity of parabens and their metabolites.

Studies in young male rats exposed during development or adolescence have shown adverse effects on sperm production and testosterone levels following exposure to parabens with longer side chains, i.e. butyl-, isobutyl-, and propylparaben. Furthermore, parabens are known to be estrogenic *in vitro* and in uterotrophic assays *in vivo*, and estrogenicity appears to increase with side chain length.

After dermal uptake, parabens are hydrolyzed and conjugated and excreted in urine. Total dermal uptake of paraben and metabolites is quite high, and has been estimated to be up to 50-80% of exposure. However, only a small amount of intact parabens can be recovered in blood and urine, and it can be estimated that approximately 2% of the applied dose is excreted in human urine in an intact form (conjugated and free). Species differences between humans and experimental animals point to higher uptake and less metabolism in human skin than in the applied rat models. This indicates higher internal doses in exposed humans than rats. However, more studies are needed to examine human levels of parabens and metabolites and to compare these levels to those obtained in experimental animal studies. It needs to be determined whether the endocrine disrupting effects seen in experimental animals are due to the (low) levels of intact parabens, or whether metabolites such as p-hydroxybenzoic acid and p-hydroxyhippuric acid may play a role.

The ability of parabens to activate the estrogen receptor may not be the only mechanism of action, as they also show anti-androgenic effects, mitochondrial toxicity and ability to elevate endogenous estrogen levels via SULT inhibition.

The estrogenic burden of free parabens and PHBA in blood may exceed the action of endogenous estradiol in childhood, when estradiol levels are very low. Additionally, the margin of safety for propyl paraben is very low when comparing worst-case exposure data to lowest observed adverse effect levels (LOAELs) from studies on young male rats or uterotrophic studies in immature mice.

There is a reason for concern for endocrine disrupting effects of parabens due to the present high human exposure levels, although uncertainties about toxicity and metabolism remain. Additional studies are needed, in particular reproduction studies on both long- and short-chain parabens, as well as extended kinetic studies.

2 Dansk resumé

Parabener er konserveringsmidler, der anvendes i en bred vifte af kosmetiske produkter, herunder produkter til børn. Desuden er methyl- og ethylparaben tilladt i visse fødevarer i begrænsede mængder. Der er dog bekymring for hormonforstyrrende effekter ved meget høje doser. Denne rapport opsummerer konklusionerne fra de seneste oversigtsartikler og giver et overblik over undersøgelser af optagelse og metabolisme og toksicitet af parabenerne og deres metabolitter.

Forsøg med unge hanrotter har vist nedsat sædproduktion og testosteron-niveau efter udsættelse for parabener med længere sidekæder, dvs butyl-, isobutyl-, og propylparaben. Desuden er parabener kendt for at være østrogene *in vitro* og stimulere uterusvækst *in vivo*, og østrogeniciteten synes at stige med sidekæde længden.

Den samlede hudoptagelse af parabener og deres metabolitter er forholdsvis høj, og kan anslås at udgøre op til 50-80% af eksponeringen. Efter hudoptagelse genfindes kun en mindre del (anslået op til 2%) af de applicerede parabener i intakt form (fri og konjugeret). Forskelle mellem mennesker og forsøgsdyr peger på en højere optagelse og mindre metabolisme i human hud end i de anvendte rotte modeller. Dette indikerer højere interne doser hos eksponerede mennesker end rotter. Dog er flere undersøgelser nødvendige for at undersøge realistiske koncentrationer af parabener og deres metabolitter hos mennesker og sammenligne disse niveauer med de niveauer, der opnås i dyreeksperimenter. Det er uklart, om de hormonforstyrrende effekter set i forsøgsdyr skyldes det (lave) niveau af intakte parabener, eller om metabolitter såsom p-hydroxybenzosyre og p-hydroxyhippur syre spiller en rolle.

Parabenernes evne til at aktivere østrogen-receptoren er ikke den eneste mulige virkningsmekanisme, da parabenerne også viser anti-androgene effekter, mitokondriel toksicitet og forøgelse af det endogene østrogen niveau ved SULT hæmning.

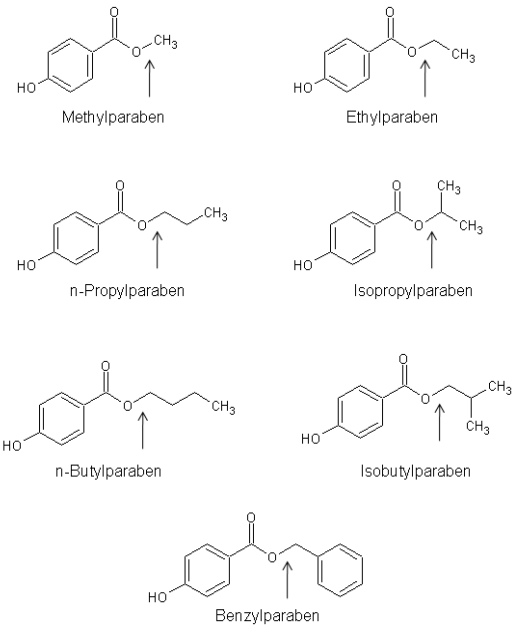
Den østrogene virkning af frie parabener og PHBA i blodet kan overstige virkningen af endogent østradiol i barndommen, hvor østradiolniveauet er meget lavt. Sikkerhedsmarginen for propylparaben er meget lav, når man sammenligner ”worst-case” eksponerings data med LOAEL fra undersøgelser i unge hanrotter eller med doser, der stimulerer uterusvækst hos unge hunmus.

De høje eksponeringsniveauer for parabener giver grund til bekymring for hormonforstyrrende effekter, om end der er en del usikkerhed forbundet med data for toksicitet og kinetik. Det mangler yderligere reproduktionsundersøgelser af både lang- og kortkædede parabener, samt udvidede kinetik studier.

3 Background

Parabens are preservatives used in a wide range of cosmetic products, including products for children. Additionally, methyl- and ethylparaben are permitted in certain foods in limited amounts. However, concern has been raised for endocrine disrupting effects at high exposure levels. Studies in young male rats have shown adverse effects on sperm production and testosterone levels following exposure to parabens with longer side chains, i.e. butyl-, isobutyl-, and propylparaben. Furthermore, parabens are known to be estrogenic *in vitro* and in uterotrophic assays *in vivo*, and estrogenicity appears to increase with side chain length.

Fig. 1. Chemical structure of parabens. Hydrolysis of the ester linkage (arrow) gives the common paraben metabolite hydroxybenzoic acid.



The current report is an update of recent literature on paraben risk assessment following up on the comprehensive review papers by Cosmetic Ingredient Review (CIR), 2008[1], and Darbre & Harvey, 2008[2].

The CIR Expert Panel, 2008, provided a review of 450 references on paraben chemistry, use, kinetics and toxicity. The CIR expert panel concludes that a high margin of safety exists for human exposure to parabens. They calculate an adult human dose of 1.2 mg/kg bw/day of multiple parabens and an infant dose of 0.3 mg/kg bw/day and compare this with a no-observed adverse effect level (NOAEL) for all parabens of 1000 mg/kg bw/day[1]. The NOAEL of 1000 mg/kg bw/day is probably (though not indicated in the CIR report) based on a study on butylparaben published in 2008 by Hoberman et al. However, the NOAEL of 1000 mg/kg bw/day in the Hoberman study may be questioned, as discussed in the toxicology section below. Additionally, it is puzzling that the CIR expert panel selects 1000 mg/kg bw/day as a NOAEL for “multiple parabens”, as they review adverse effects of butyl- , isobutyl-, propyl-, ethyl-, and methylparaben at doses below 1000 mg/kg bw/day.

Darbre and Harvey, 2008, reviewed more than 150 references on paraben toxicity, kinetics and exposure, and discuss mainly the burden of parabens on breast cancer risk due to estrogenic effects and conclude

that there is a need for a detailed evaluation of the effects of mixtures of parabens and other environmental contaminants on breast cancer and male reproductive function[2].

A risk assessment of the Scientific Committee on Consumer Products (SCCP) of the European Union in 2005 concluded that methylparaben and ethylparaben may be safely used in cosmetics at concentrations up to 0.4%, but that data for propylparaben, isopropylparaben, butylparaben and isobutylparaben were insufficient. An acceptable daily intake (ADI) of 10 mg/kg bw/day could be determined for methyl- and ethylparaben, but no ADI could be determined for the other parabens[3]. The SCCP requested reproductive and developmental toxicity studies and *in vitro* studies on percutaneous absorption.

A recent publication by Cowan-Ellsberry et al. of The Procter & Gamble Company, 2009, uses refined aggregate exposure estimates for paraben exposure and calculated a lower aggregate exposure than would be calculated from simple addition[4]. They compared exposure data to biomonitoring data and found that aggregate exposure estimates were 2 to 92 fold higher than exposure estimated from biomonitoring data[4]. They concluded that even these conservative aggregate exposure estimates were below an ADI of 10 mg/kg bw/day. However, this ADI was from SCCP and, as mentioned above, not intended to include all parabens, but for methylparaben and ethylparaben only [3].

In addition to the controversy on selection of NOAELs for risk assessment, there are conflicting data on the dermal absorption and metabolism of parabens and how to interpret these data in relation to human risk assessment. This update will provide a review of the knowledge on paraben uptake and metabolism, and address data needs regarding paraben kinetics and toxicity.

4 Paraben uptake

4.1 Metabolic pathways

Dermally applied parabens are taken up by skin and metabolized by esterases. Uptake depends on the ester chain length and the formulation. In general, skin permeation decreases with increasing chain length. Lipid solubilizers reduce percutaneous absorption, while penetration enhancers increase penetration (reviewed in CIR, 2008)[1]. After uptake, parabens and their metabolites are conjugated and excreted in urine and bile. The main metabolite is para-hydroxybenzoic acid, PHBA, and a large proportion of PHBA is excreted as p-hydroxyhippuric acid (PHHA, the glycine conjugate of PHBA). Following oral exposure, parabens are metabolized by esterases in intestine and liver, and in addition to the urinary excretion of parabens taken up in blood, some excretion occurs in bile and feces.

4.2 Uptake and metabolism of parabens in humans

4.2.1 Blood levels

Parabens are excreted in urine as glycine, glucuronide or sulphate conjugates of the parent compound or of the metabolite PHBA. Data obtained from chronic administration studies indicate that parabens do not accumulate in the body, and serum concentrations of parabens, even after intravenous administration, quickly decline and remain low.

Parabens and their metabolites can be measured in human blood and urine. Ye et al., 2008 measured methyl-, ethyl- and propylparaben in human serum samples using on-line SPE-HPLC-MS/MS. A mean level

of 0.4 ng/ml of free propylparaben was detected with a maximum value of 2.3 ng/ml. Enzyme treatment was performed to measure also the amount of conjugated species, and total propylparaben was measured to 8 ng/ml (mean) with a maximum level of 67.4 ng/ml, and 87% was detected as conjugated species. For methyl- and ethylparaben, mean levels of 42.4 and 0.6 ng/ml were measured in serum and 90% and 100% were conjugated, respectively[5].

Janjua et al., 2007, applied a cream containing 2% (800 mg/person; 10 mg/kg bw) of butylparaben (together with 2% diethylphthalate and 2% diethylmethylphthalate) to human male volunteers[6]. Serum levels of parent compound were determined by HPLC with MS detection. The peak blood concentration (at 3 h post dose) of free, unconjugated butylparaben was 135 µg/l corresponding to 700 nM. The authors estimated that 0.8 mg of butylparaben is in circulation at the time of peak concentration corresponding to 0,1% of parent compound[6]. However, the total amount taken up is likely to be much larger.

During the following 4 days, test persons were exposed once daily, and blood levels reached approximately 20 µg/L corresponding to the dose at 24 hours after the first dose. Thus, no accumulation was expected with the applied dose once daily[6].

In the studies by Ye et al., 2008, measured levels of free propylparaben (2,3 ng/ml) were lower, but in the same range as the levels of free butylparaben (20 ng/ml at 24h post dose) measured by Janjua et al., 2007. However, a realistic range of free serum paraben levels in a human population has still not been published, as Ye et al., 2008, only studied five serum samples from a serum bank.

Heim et al., 1957, administered 10 or 20 mg/kg of ethylparaben orally to humans and measured levels of free ester and PHBA in serum[7]. They did not detect any free ethylparaben, but peak values of 2 and 3.5 µg/ml PHBA in serum corresponding to 1% of the administered dose. However, these values cannot be used to estimate total uptake levels due to conversion of the paraben to other metabolites and distribution to other organs.

4.2.2 Urinary levels

Urinary measurements in humans can be used for estimation of uptake. Janjua et al., 2008, analyzed levels of free and conjugated paraben in urine of the test persons described above[8]. A mean of 2.5 mg/person/day of free and conjugated (glucuronidated) butylparaben was detected indicating a recovery of 2.5 mg of 800 mg = 0.3%. Maximal recovery was 0.9%. Total uptake was probably larger, as it is stated that more than 50% of parabens are eliminated in a sulphated form, which was not measured. A systemic uptake of up to 2% (free and conjugated) butylparaben can be suggested. In addition, a large percentage is present as metabolites, mainly free and conjugated p-hydroxybenzoic acid. As will be discussed in the following section, animal studies show that more than 90% of administered paraben may be present as metabolites, and the same may apply to humans.

Ye et al., 2006, measured parabens in urinary samples and found 95% of methylparaben and 98% of propylparaben in a conjugated form (sulphated and glucuronidated)[9].

Table 1 lists findings in experimental studies in humans and laboratory animals on uptake of parabens and their metabolites. Mainly data on free and conjugated parabens are available, whereas data on metabolite levels in human blood and urine are lacking and will be necessary to determine total uptake levels and metabolic pathways.

Table 1. *Uptake of parabens, %*	*Intact parent compound*	*PHBA after paraben administration*	*Parent compound + metabolites total*
Human dermal *in vivo* [6;8]	0.1% at peak (free ButP, blood) 0.9-2% ButP (urine, free and conjugated)		
Human oral *in vivo*[7]	"not detectable"	1% of dose in serum at peak	
Dog oral *in vivo*[7]	"Low" serum levels (5 µg/ml of EthP)	"high" serum levels (55 µg/ml)	
Rabbit oral *in vivo* [10]	0.2-0.9% (urine)	26-36% (urine)	70-94% (urine)
Rat oral *in vivo* (Derache and Gourdon, 1963 in [1])	"not detectable"		67-75% PHBA and parabens in total (MetP, EthP, ProP, urine)
Rat dermal *in vivo*[11] (ButP)			46% (4 h, urine)[11]
Rat intraduodenal *in vivo*[7] (2 mg/kg of EthP)	Low but detectable levels of EthP Bioavailability: 6.7%	11 times higher than intact EthP	PHHA levels 61 times higher than intact EthP
Human dermal *in vitro*	0.2% (full thickness skin[12])	15,2% (full thickness skin[12])	14.9% (full thickness skin[12])
	60% MetP, 49,7% ButP (Fasano 2004 ref in [1])	35% and 32.8% after MetP and ButP admin (Fasano 2004 in [1])	21% after ButP admin, full thickness skin (Fasano 2004 in [1])
	33, 44, 37, 37 and 17% of MetP, EthP, ProP, ButP and BzP [13]		
Rat dermal *in vitro*	6% ButP, 35% ProP [14] 4% ButP, 30% ProP [15]		57% after ButP admin [11]
	24% MetP; 5.5% ButP (Fasano 2004 in [1])	54% after MetP, 52% after ButP administration (Fasano 2004 in [1])	
Rabbit dermal *in vitro*	60% MetP, 40% EthP, 20% ProP [16]		

4.3 Uptake and metabolism of parabens in experimental animals

4.3.1 Blood levels

Data on the uptake of parabens in experimental animals may be used either as a model for human uptake (e.g. dermal) or to calculate the "internal dose" of parent compounds following e.g. oral dosing in toxicity studies.

Despite the comprehensive literature on metabolism of parabens in experimental animals, very few studies report *serum* levels of parabens in rats following oral or subcutaneous exposure, which are the exposure routes used in toxicity studies.

In Frederiksen et al., 2008, pregnant rats were subcutaneously exposed to 100, 200 and 400 mg/kg bw/day of ethylparaben and butylparaben[17]. Parabens were measured in maternal plasma, embryonic fluid, placenta, fetal liver and fetal carcass by LC-MS/MS. The concentration of butylparaben (deconjugated, i.e. free + glucuronidated and sulphated parent compound) was higher in embryonic fluid (2000 ng/ml), fetal carcass (370 ng/g) and liver (260-280 ng/g) than in maternal plasma (250 ng/ml, corresponding to approximately 125 ng/ml in whole blood). This indicates that parabens do cross the placenta and are taken up by fetuses, and that some degree of up concentration takes place in comparison to maternal blood. However, these measurements were done at only one time point after dosing, and further studies would be required to elaborate on the distribution of parabens and their metabolites. The uptake of ethylparaben appeared to be higher than the uptake of butylparaben as a 10-fold higher blood and tissue levels of ethylparaben than butylparaben were observed[17].

Heim et al., 1957, administered 25, 100 or 500 mg/kg of ethylparaben orally to dogs and measured levels of free ester and PHBA in serum[7]. They did not detect any free ethylparaben at the two lowest doses but a peak level of 5 µg/ml at the highest dose. At the highest dose, the peak concentration of PHBA was 55 µg/ml, i.e. the ratio of free ester to PHBA was 1:11. No other metabolites were measured. The finding of free parabens in the highest dose only corresponds with the lack of detectable paraben levels in serum after exposure to 100 mg/kg bw/day in the rat studies by Derache and Gourdon, 1963 (reviewed in [1]) and by the European Cosmetics Association [18]. In the study by Derache and Gourdon, 1963, no unchanged paraben was detected by paper chromatography following oral exposure of rats to 100 mg of methyl-, ethyl- or propylparaben[1].

Kiwada et al., 1980, compared the metabolism of PHBA and ethylparaben in rats and found relatively high levels of the glycine conjugate p-hydroxyhippuric acid (PHHA). In fact, the blood concentration of PHHA was higher after ethylparaben exposure than after PHBA exposure[19]. They concluded that metabolism may occur through alternative routes than conversion of ethylparaben to PHBA and then to PHHA, and assumed a direct metabolic route from ethylparaben to PHHA. The blood AUC (total uptake in blood) for PHHA was 2.5 times higher than for PHBA following intravenous ethylparaben administration and 5.4 times higher following intraduodenal administration. With intraduodenal administration of ethylparaben, PHHA is the main metabolite with a blood AUC of 54.8 mg/ml per hour, whereas the AUC for ethylparaben is 0.9 mg/ml per hour, i.e. 61 times lower[19].

With intravenous ethylparaben administration the blood AUC of p-hydroxyhippuric (pHHA) acid was 2.8 times higher than after PHBA administration, indicating higher blood levels of this metabolite [19]. With intraduodenal administration of PHBA the AUC for pHHA was closer to the AUC for ethylparaben (1.7 times higher for ethylparaben than PHBA) due to direct conjugation of PHBA in the liver.

The bioavailability of free ethylparaben with intraduodenal administration can be calculated as: $(AUC_{id}/dose_{id}) / (AUC_{iv}/dose_{iv}) = AUC_{id} / AUC_{iv} = 0.9/ 13.5 = 6.7$ %. This value could in the absence of specific data be used for estimating the internal dose in animal studies exposed orally.

4.3.2 Urinary levels

Metabolites of methylparaben have been described in rabbit urine following gastric intubation (Tsukamoto and Terada, 1960, 1962, reviewed in [1]). They identified p-hydroxy benzoic acid (PHBA), p-hydroxyhippuric acid (PHHA), p-carboxyphenyl glucuronide, p-hydroxybenzoyl glucuronide, and p-carboxyphenyl sulphate. They report that 0.2-0.9% of unchanged ester was excreted, and that the urinary excretion of PHBA was slower with increasing carbon chain length of the paraben alkyl group. Tsukamoto and Terada (1964) compared the metabolism of PHBA and parabens in rabbits and found that the urinary excretion of free PHBA is less after paraben exposure than after PHBA exposure, and that urinary excretion of free PHBA was lower with longer chain lengths[10] (see Figure 2). Some variation applies to these data.

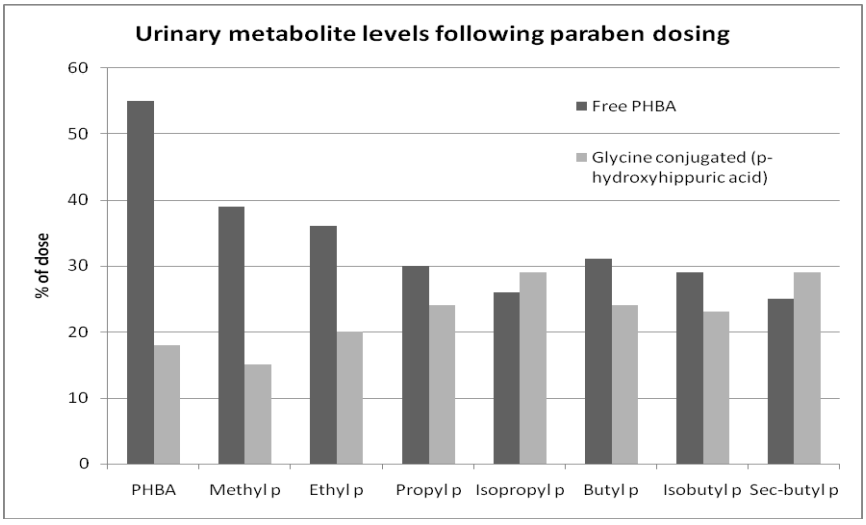


Figure 2. Urinary excretion of PHBA and PHHA in rats exposed to the parabens listed on the horizontal axis. Based on Tsukamoto and Terada, 1964[10]

These results point to a difference in the metabolism of PHBA and particularly the long-chain parabens. The excretion of glycine-conjugated PHBA appears to be slightly higher with increasing ester chain lengths. It is possible that the difference in toxicity of these compounds is related to differences in metabolism.

The total urinary levels of parabens and metabolites in that study were 70 to 94% of the orally administered dose[10]. Yamashita et al., 1994, reported urinary excretion of 46% of a dermally administered dose of butylparaben and calculated an absorption of 51% [11].

COLIPA measured metabolites in blood and urine in a study on dermal, subcutaneous and oral exposure to C14 labelled parabens in rats, and reported 14 to 27% absorbtion with all C14-label excreted as PHBA [20]. Data from this study are not available, but may be useful in estimating total uptake and metabolism, although there appears to be some discrepancy with other studies in which considerable levels of other metabolites than PHBA have been found.

Table 1 summarizes the data on uptake and metabolism of parabens *in vivo* and *in vitro*. Overall, the *in vivo* uptake of intact paraben is extremely low compared to metabolite levels. Estimates of uptake of the dermally applied dose range from 46 to 94% based on urinary excretion levels of metabolites.

4.4 *In vitro* studies on human and animal uptake and metabolism

4.4.1 Uptake *in vitro*

The figure of 0.1% free, unhydrolyzed paraben in blood at peak following human dermal exposure (described above), corresponds well to the 0.2% uptake of free, unhydrolyzed paraben estimated from an *in vitro* study on dermal penetration through human full thickness skin conducted by Fasano et al., 2005[12]. In that study, 15-21% of C14-labelled butylparaben was taken up, with the larger part (more than 99%) being PHBA.

Other studies on human skin *in vitro* showed higher uptake of unhydrolyzed parabens. Jewell et al., 2007, measured percutaneous penetration of 37% butylparaben and propyl paraben absorbed through human skin to receptor medium after 24 hours. 50% and 44% remained in skin at 24 hours. Less absorption was seen for minipig skin in that study[13].

Several *in vitro* studies on dermal penetration through rat skin show uptake levels from 6 to 35 % [14-16]. However, no studies have shown detectable levels of free, unhydrolyzed parabens in rat serum *in vivo*, which must be explained by a rapid metabolism.

In a study by Pedersen et al. (2007) a rabbit skin model system was used to assess the skin permeation and retention of methyl-, ethyl and propyl paraben from three commercial cosmetic creams[16]. The conclusion from the results they obtained was that parabens permeate across the skin from cosmetic creams, and the extent of penetration depends more on paraben characteristics such as solubility and lipophilicity (permeation deceasing with decreasing solubility and increasing lipophilicity) than on the composition of the formulation.

Overall, the uptake *in vitro* appears to depend on the species used in the assay, and for the human studies also whether full thickness skin has been used. The total amount of parent compound and metabolites penetrating skin *in vitro* ranges from 15 to 57%. This is slightly lower than the 46 to 94% dermal uptake *in vivo* estimated from urine measurements, but large uncertainties apply to these figures. In their risk assessments, CIR and Cowan-Ellsbury use 50 and 80%, respectively as conservative estimates of paraben penetration of human skin [1;4].

SCCP, 2006, have evaluated data from the studies by Fasano et al., 2005[12] and 2004 and list a number of shortcomings leading to the conclusion that “interpretation of the obtained results remains questionable”[21].

4.4.2 *In vitro* metabolism

Metabolism studies are performed in microsomes of liver and skin of human and rat origin. In rat liver, paraben hydrolysis was much slower than in human liver, rat liver, and rat skin [22]. Butylparaben was degraded faster in rat skin than in human skin[22], indicating that human dermal uptake of the parent compound is larger than expeted from a rat skin-model. Williams et al, 2008, found that metabolism studies with flow showed less metabolism than *in vitro* studies. They concluded that “although human skin

has capacity to metabolize parabens, the amount of local metabolism in the skin during dermal penetration *in vivo* would be low" [23].

These data indicate that oral dosing of rats will give a low internal dose due to the high degree of metabolization compared to human oral or dermal exposure. Furthermore, (assuming that these assays are optimal and provide a good estimate of dermal versus oral uptake) this implies that if calculations to compare human exposure and rat effect levels use a correction for low uptake of intact parabens in human skin, these calculations must be supplied with an even larger correction for uptake in the rat.

Table 2. Microsomal metabolism	*Human skin nmol/min/ mg*	*Human liver nmol/min/ mg*	*Human skin:liver*	*Rat skin nmol/min/ mg*	*Rat liver nmol/min/ m)*	*Rat skin:liver*	*Human skin: rat liver*
ButP[22]	0,05	15	1:300	125	150	1:1	1:3000
ButP[24]		25					
ButP [13]	100	30000	1:300				
Other esterase substrates[25]	80	3700		1500	5500	1:4	1:700

4.5 Conclusion on uptake and metabolism

Overall, these studies reveal rather low levels of free parabens in blood of exposed humans and no free parabens in experimental animals. However, total levels of metabolites and parent compounds excreted in urine of orally and dermally exposed rats and rabbits are high, indicating that parabens and/or their metabolites are taken up in considerable amounts, but rapidly metabolized and excreted. In their exposure estimate, Cowan-Ellsberry et al., 2009, use a conservative estimate of 80% total uptake of parent compound or metabolite based on dermal penetration levels from 15 to 75% in various studies *in vivo* [4].

Apparently, low peak concentrations (0.1% of dose) of unconjugated, intact parabens can be detected in human serum. To get estimates of human uptake levels, kinetic studies assessing total "area under the curve" (AUC) values of free and metabolized parabens in blood after dermal or oral uptake should be compared to AUC values after intravenous exposure. AUC values determine the total blood level over time and are proportional to uptake levels.Blood levels of parent compounds and estradiol levels will be compared in section 8 and related to estrogenic potencies of these compounds.

According to the studies by Fasano et al., 2004 (referred in [1]), uptake of intact parabens in human skin *in vitro* is higher than in rat skin. This is in accordance with the low levels of unconjugated, unhydrolyzed parabens detected in dermally exposed humans, but no detectable levels in rats. In their later study, Fasano et al., 2005, find very low uptake of intact parabens in human full thickness skin[12]. Further studies using up-to-date methods may elaborate on differences between rats and humans.

After uptake into the blood stream, distribution to organs occurs, but due to the rapid hydrolyzation and conjugation, concentrations of parent compounds also are expected to be very low at the active sites in target organs. It can be estimated that up to 2% of intact (free and conjugated) butylparaben is excreted in urine of dermally exposed humans indicating a systemic dose of butylparaben of around 2%.

Nevertheless, it is obvious to consider whether the toxicity of parabens is due to their metabolites, and further studies determining metabolite concentrations in blood and target organs are required.

5 Paraben toxicity *in vivo*

5.1 Male effects

In the risk assessment of propyl- and butylparaben, studies by Oishi from 2001 and 2002 are central as they show effects on sperm count and testosterone levels after 4 and 8 weeks dietary exposure of rats to butyl- and propylparaben, respectively[26;27].

In the study on propylparaben, daily sperm production (testis sperm production) was reduced in all three dose levels of approximately 10, 100 and 1000 mg/kg bw/day. At 100 mg/kg bw/day, epididymal sperm count was affected in a dose-related manner. Serum testosterone levels were reduced in a dose-related manner in all dose groups, but only statistically significant at 1000 mg/kg bw/day. Body weight was reduced at 1000 mg/kg bw/day[27]. This indicates a LOAEL of 10 mg/kg bw/day for propylparaben.

In the study by Oishi, 2001, daily sperm production (testis sperm counts) as well as epididymal cauda sperm counts were reduced in a dose-related manner in all applied doses of approximately 10, 100 and 1000 mg/kg bw/day of butylparaben[26]. Serum testosterone was reduced at 100 and 1000 mg/kg bw/day showing a dose-response relationship. Body weight was not statistically significantly reduced. Relative epidiymis weight was reduced at 100 and 1000 mg/kg bw/day with a dose-response relationship. Absolute, but not relative weight of the seminal vesicle was reduced at 1000 mg/kg. Thus, the LOAEL was 10 mg/kg bw/day, and no NOAEL could be determined.

Hoberman et al., 2008, performed a repeat study of the study by Oishi from 2001 by exposing young male rats in the diet to 10, 100 and 1000 mg/kg bw/day of butylparaben[28]. They reported “no adverse effects” at all dose levels concluding a NOAEL of 1000 mg/kg bw/day. However, serum testosterone was reduced significantly after 3 weeks of dosing at 100 and 1000 mg/kg bw/day (see Figure 3). In the paper is stated that this was due to two high outliers in the control group. Examination of raw data reveals that the effect is still statistically significant in the highest dose group after removing the two outliers from the control group. The effects on testosterone levels at week 3 may be important to the masculinization of the rats taking place at this age (prepuberty, 6 weeks of age) and may be regarded as adverse. Additionally, in all exposed groups some animals had lower daily sperm production than the lowest control values (see Figure 4). Therefore, it cannot be ruled out that some animals are affected by dosing, although there was no statistically significant difference between group means.

The European Union Scientific Committee on Consumer Products (SCCP) have evaluated the study report on this butylparaben study and conclude that due to several shortcomings the study “cannot be considered as scientifically valid” [21]. The SCCP remarks that body weights are more divergent than accepted in classical toxicity studies, and that hormone measurements have large standard deviations and no information of time of blood collection. Additionally, animals display unexpected clinical symptoms and the SCCP question the general health of the animals. The SCCP also question the validity of a positive control study submitted together with the study report from the butylparaben study[21].

Figure 3. Testosterone levels at week 3 after start of dosing (6 weeks of age) in rats exposed to 10, 100 and 1000 mg/kg bw/day of butylparaben[28]. According to the paper, testosterone levels were reduced significantly in the two highest dose group when including two high outliers. When removing these two outliers, the reduction in testosterone levels was still statistically significant in the highest dose group when we used an ANOVA followed by Dunnett's post hoc test (p=0.009).

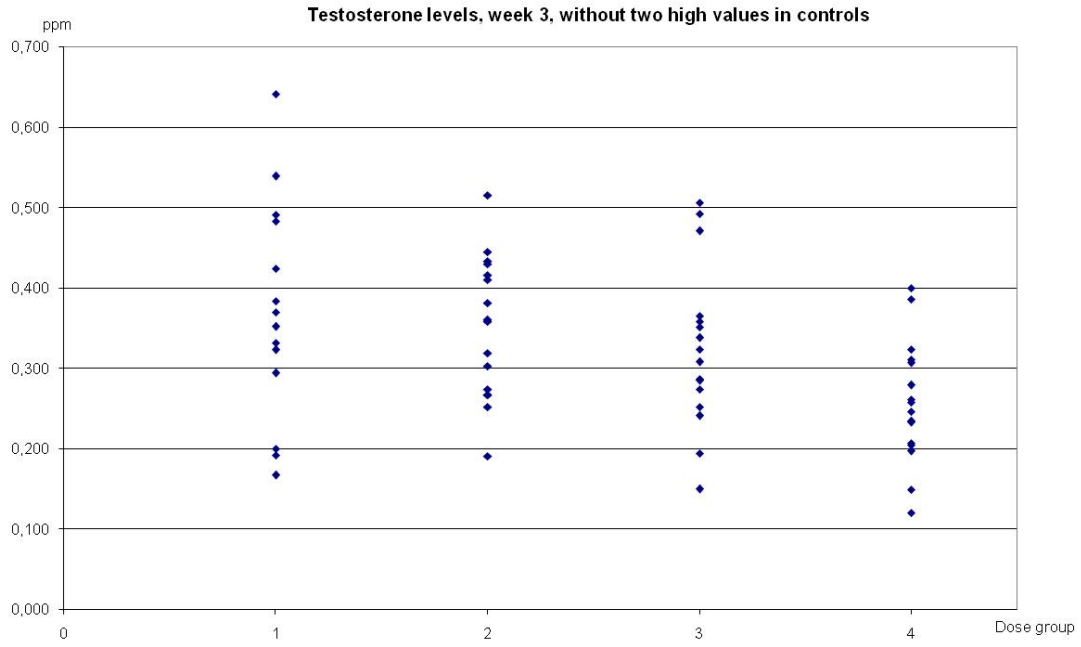
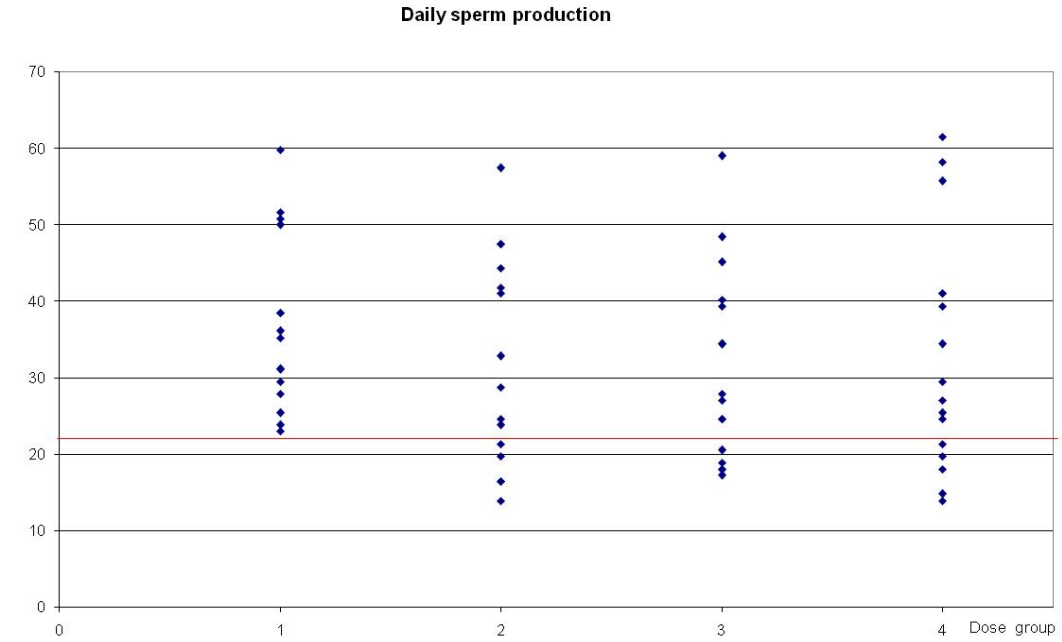


Figure 4. Daily sperm production at the end of dosing (11 weeks of age) in rats exposed to 10, 100 and 1000 mg/kg bw/day of butylparaben[28]. In each group exposed to butylparaben, 5 animals had sperm production values below the lowest control value.



Hoberman et al., 2008, also investigated the effects of methylparaben in a similar study design, except no hormone measurements were reported[28]. In that study, a statistically significant increase in the number of abnormal sperm was reported in the two highest dose groups, and the testicular spermatid concentration appeared dose-dependently decreased (to 77% of control level), although this was not statistically significant[28]. Further investigation of the study report reveals that also the number of normal sperm is reduced, but only the summary report was available and raw data were not available. Only absolute organ weights are reported in Hoberman et al., 2008, although the study report reveals a statistically significant increase in relative, but not absolute liver weight. Prostate weight was increased to 132% of control levels in the highest dose group, but this was not statistically significant[28]. An increase in prostate weight after administration of estrogenic compounds has been reported in other studies[29;30]. The authors conclude that no dose related effects are observed, but the validity of this statement cannot be evaluated without the full data available. SCCP have evaluated this methylparaben study based on raw data and conclude that the study is not scientifically valid and may "undermine the decision taken earlier for methyl paraben" of an ADI of 10 mg/kg bw/day based on a NOAEL of 1000 mg/kg bw/day.

Oishi, 2004, found no adverse effects of 100 and 1000 mg/kg bw/day of methyl- and ethylparaben in young male rats exposed in the diet for 8 weeks[31].

Kang et al., 2002, investigated effects of subcutaneous exposure to 100 or 200 mg/kg bw/day of butylparaben in utero and during lactation[32]. Reduced sperm count was observed in both dose groups, and no NOAEL could be determined. Testis and prostate weights relative to brain weights were reduced, but not in a dose-related manner and the authors did not list organ weights relative to body weights[32].

Taxvig et al., 2008, studied the effects of subcutaneous exposure of pregnant rats (gestation day 7 to 21) to 200 and 400 mg/kg bw/day of butylparaben or ethylparaben and found no changes in anogenital distance in male fetuses and no alteration of fetal testosterone production[33].

Daston et al., 2004, found no developmental effects of 10, 100 or 1000 mg/kg bw/day of butylparaben administered by oral gavage from gestation day 6 to 19, although maternal weight gain was reduced in the highest dose group[34]. Endocrine related endpoints were not investigated in that study.

No studies on effects of subcutaneous exposure of young males have been reported. Subcutaneous exposure is relevant for paraben studies, as the first-pass effect (metabolism in liver) is avoided similarly to human exposure to parabens via dermal application.

5.2 Uterotrophic effects of parabens and PHBA

CIR list effects of parabens and PHBA in uterotrophic studies in immature or ovariectomized mice and rats, and an extended version of this list is presented in Table 3. Overall, it can be concluded that all parabens and PHBA had uterotrophic effects in at least one study. However, negative results were seen for methyl-, ethyl-, propyl- and butylparaben and PHBA in at least one other study.

Table 3. Summary of results of uterotrophic assays. Modified from [1]. Route: subcutaneous (SC) unless otherwise stated.

Study	Response in immature rats (effective doses in mg/kg)	Response in immature mice (effective doses in mg/kg)	Response in ovariectomized mice (effective doses in mg/kg)	E2:chemical potency ratio (from CIR[1])
		p-hydroxybenzoic acid		
Lemini 2003[35], s.c.	No effect at 50 and 150	LOEL 150 NOEL 50		500-2500
Lemini 1997[35], s.c.		LOEL 5 NOEL 0.5	LOEL 5 NOEL 0.5	1000
Hossaini 2000 [36]	No effect at 5	No effect at 5 or 100		
Twomey 2000		No increase up to 100		
		Methylparaben		
Routledge 1998[37]	No effect up to 80, sc No effect up to 800, oral			
Hossaini 2000[36]		No effect at 100 (sc) No effect at 1, 10 and 100 (oral)		
Lemini 2003[35], s.c.	LOEL 55 NOEL 16.5	LOEL 16.5 NOEL 5.5	LOEL 165 NOEL 55	1041/3448
Lemini 2004[38]			LOEL 55	5000/20000
		Ethylparaben		
Hossaini 2000 [36]		No effect at 100 (sc) No effect at 1000 (oral)		
Lemini 2003[35], s.c.	LOEL 180 NOEL 60	LOEL 60 NOEL 18	LOEL 18 NOEL 6	345/12500/
Lemini 2004[38]			LOEL 60	3333/25000
		Propylparaben		
Hossaini 2000 [36]		No effect at 100 (sc) No effect at 1, 10 and 100 (oral)		
Lemini 2003[35], s.c.	LOEL 65 NOEL 20	LOEL 20 NOEL 6.5	LOEL 20 NOEL 6.5	1600-1800/5263
Lemini 2004[38]			LOEL 65	3333/20000
		Isopropylparaben		
Vo 2009[39]	LOEL 1000 NOEL 250			
		Butylparaben		
Routledge 1998[37]	LOEL 400 NOEL 200			15000
Hossaini 2000 [36]	LOEL 100, sc	No effect at 100		6000
Lemini 2003[35]	LOEL 70 NOEL 20	LOEL 7 NOEL 0.7	LOEL 21 NOEL 7	436/16666
Lemini 2004[38]			LOEL 70	5000/11111
Shaw 2009[39]			No effect up to 1000	
Vo 2009 [39]	LOEL 1000 NOEL 250			
		Isobutylparaben		
Darbre 2002[40]		LOEL 72		240000-2400000
Koda 2005[41]	LOEL 250 NOEL 100 (ovx rat)			4000000
Vo 2009[42]	LOEL 1000 NOEL 250			
		Benzylparaben		
Darbre 2003[43]		LOEL 2500 (topical) NOEL 1000 (topical)		330000-3300000

Negative results for parabens were found in uterotrophic assays in immature B6D2F1 mice using subcutaneous and oral doses of up to 100 mg/kg bw/day [36]. In that study, only the positive control estradiol

benzoate revealed positive effects. For PHBA, negative results were seen in immature rats and mice subcutaneously exposed to 5 mg PHBA/kg bw/day [36].

In addition to the studies listed in the CIR report, a study by Lemini et al (2003) showed slight uterotrophic effects of PHBA at 150 but not 50 mg/kg bw/day in immature mice, whereas no effects were seen in immature Wistar rats[44]. In that study, the uterotrophic effects of parabens were compared using immature rats and mice and ovariectomized mice and concluded that the mouse immature model was the most sensitive. The uterotrophic effects of methyl-, ethyl-, propyl-, and butylparaben were within the same range, and the authors concluded that the activity increases with the chain length, but this was not supported by their data[44]. PHBA had weaker effects than the parabens, and it was estimated that PHBA was 500 to 2500 times less potent than E_2 in that assay. In the immature mouse assay, ethyl- and butylparaben were 346 and 437-fold less potent than E_2, respectively, when calculated from the lowest active doses of parabens [44]. Potencies were from 3000 to 30000-fold less potent than E_2 when comparing the higher doses of parabens, as also listed in Table 3 [1]. NOELs in the immature mouse assay were from 0.6 to 6.5 mg/kg bw/day for parabens and 50 mg/kg bw/day for PHBA (LOELs were 7 to 60 mg/kg for parabens and 150 for PHBA)[44]. These LOELs correspond to the LOAELs of 10 mg/kg bw/day for butylparaben and propylparabens in the studies by Oishi [26;27].

It is noteworthy that the listed potency estimates are very variable between studies and when calculations are based on different doses in each study. In order to estimate potency differences correctly it would be necessary to have full dose-response curves for not only the test compounds but also E_2. However, most of the listed studies only include one dose of E_2 and therefore are rather imprecise.

Since the publication of the CIR review in 2008, a few more studies have been published, including a study by Shaw et al., 2009, in which no uterotrophic effect was reported in ovariectomized mice administered up to 35 mg/mouse (approximately 1000 mg/kg bw/day) of butylparaben subcutaneously[39]. However, the statistical analysis appears to be rather conservative and it is possible that a statistically significant effect would be seen in the highest dose group if evaluated with statistical methods applied in the other studies. Vo et al., 2009, found increased uterus weight with exposure to 1000 mg/kg bw/day of isopropyl-, butyl-, and isobutylparaben in immature female SD rats[42]. With isobutylparaben, a statistically significant increase was also seen at 250 mg/kg bw/day.

The review by CIR, 2008, describes a study by Twomey, 2000, investigating uterotrophic effects of PHBA in immature CD-1 mice. Doses administered were 0.5, 5.0, 50 and 100 mg/kg bw/day by subcutaneous injection for 3 consecutive days (PND20 to 22). The CIR review (p. 48) describes significantly *decreased* uterus weights compared to controls although no dose-response relationship was reported, and it is not stated at which doses the effects were seen[1]. However, data were not available for the current review.

Overall, all parabens and PHBA can be regarded as estrogenic *in vivo* due to their uterotrophic effects. The potency differences between the parabens do not seem to be strictly related to ester chain length in the uterotrophic assay, although such a relationship appears to exist in *in vitro* studies[37;45].
It should be considered that metabolism overload may occur with subcutaneous exposure resulting in high serum levels of unmetabolized parabens, i.e. metabolic capacity is exceeded. However, this may also be relevant for the realistic application of parabens in lotions, as exposure is likely to be through acute, high doses rather than application of lower doses over a long time.

In vitro studies on toxicity of parabens and PHBA

5.3 Estrogenic effects

In general, all the widely used parabens have been shown to possess estrogenic activity to different extent in different assay systems *in vitro* and *in vivo*. In Table 4, based on a review by Darbre and Harvey, 2008, the majority of the listed studies report estrogen agonist activity, and only 1 out of 25 estrogenicity assays *in vitro* studies report negative findings [2]. The estrogenic activity of parabens is known to increase with increasing chain length and with branching of the alkyl chain [37;45]. In addition, studies have shown that the common metabolite of the parabens, PHBA, also possesses estrogenic activity in both *in vitro* and *in vivo* assays[44;46]. Thus, removal of the alkyl grouping reduces activity but does not remove all estrogenicity [2].

Van Meeuwen et al., 2008, studied the estrogenic and aromatase inhibitory effects *in vitro* of several cosmetic additives, including parabens and PHBA[47]. The parabens were tested both alone and in different combinations. The individual parabens, but not PHBA, showed estrogenic effect in the MCF-7 cell proliferation assay, and the maximally induced cell proliferation could be fully blocked by the ER antagonist ICI 182780. The results from the different mixtures of parabens as well as the parabens in combination with E_2 showed additive effects between the different parabens and between the parabens and E_2[47]. Furthermore, all the tested parabens were found to significantly inhibit aromatase activity, although to different extent, whereas PHBA had no significant effect on aromatase activity. The observed aromatase inhibitory properties occurred at concentrations that were within one order of magnitude of the effective concentration inducing cell proliferation in MCF-7 cells [47]. Thus, at estrogenic effective levels of parabens, aromatase inhibition could also be expected to some extent. As aromatase is the enzyme responsible for conversion of androgens into estrogens, its inhibition could be considered as an opposite, i.e. anti-estrogenic effect, to the cell proliferation occurring in estrogen responsive tumor cells. In a human situation, inhibition of aromatase could therefore diminish estrogenic effects.

The authors estimated cumulative estradiol equivalents (EEQ) for human internal systemic exposure to various compounds acting through the estrogen receptor and brought this total EEQ value into relation to endogenous and therapeutic levels of E2 and EE[47]. Based on that, they concluded that the obtained total EEQ value is unlikely to cause adverse effects in humans. Importantly, these comparisons were compared to endogenous and therapeutic levels of estrogens in adult women, whereas they did not consider estrogen levels in children. Although the authors did not find estrogenic effects of PHBA in the MCF-7 cell proliferation assay, the authors expressed concern due to the possible estrogenic effects of PHBA as reported by other authors[46] and due to relatively high human PHBA levels reported in a Japanese study[47]. An estimate of the estrogenic capacity of paraben exposure compared to estradiol levels in children will be discussed in section 7.

Darbre and Harvey, 2008, discuss the question whether parabens should be termed weak estrogens[2]. The ability of parabens to mimic estrogen action is well documented, but different studies have shown that parabens have lower binding affinity to ER than some other estrogenic ligands and parabens are often termed “weak estrogens”. However, the lower binding affinity to ER does not result in reduced efficacy if sufficient concentration of paraben is present. With sufficient concentrations, the parabens gave responses in whole cell assays in terms of increased gene expression and cell proliferation in human breast cancer

cells of the same magnitude as 17β-estradiol [40;43;45]. As discussed by Darbre and Harvey, 2008, parabens are not partial agonists, as might be implied by the term 'weak', but give full agonist responses in whole cells. It is possible that in cell-based assays the parabens might achieve full agonist response at lower concentrations given more time[2]. This parameter has not been considered in most published studies on parabens. The principle that partial agonist effects can be enhanced over a longer time period has been shown in the case of the estrogen agonist properties of triclosan [48]. Darbre and Harvey, 2008, therefore suggest that this should be repeated with the parabens, as it is highly relevant to environmental situations where the compound would be present over the long term and not only for a set time frame. As a result, Darbre and Harvey, 2008, recommend that the operational label of 'weak estrogen' be reconsidered.

Table 4 Summary of *in vitro* studies published on the oestrogenic activity of parabens. Based on [2]

Paraben	*Result in vitro*
Methylparaben	+ ve (yeast + receptor binding) [37;49-51] + ve (human MCF7) [45;46;50;52] - ve (rat uterus receptor binding) [44] + ve (rat uterus receptor binding) [53]
Ethylparaben	+ ve (yeast + receptor binding) [37;49-51] + ve (human MCF7) [45;50;52;54] + ve (rat uterus receptor binding) [44;53] + ve (human HeLa overexpressing ER) [55]
***n*-Propylparaben**	+ ve (yeast + receptor binding) [37;49-51] + ve (human MCF7) [45;50;52;54] + ve (rat uterus receptor binding) [53] [44] + ve (human HeLa overexpressing ER) [55]
***n*-Butylparaben**	+ ve (yeast + receptor binding) [37;49-51] + ve (human MCF7) [45;46;50;52;54] + ve (rat uterus receptor binding) [44;53] + ve (human HeLa overexpressing ER) [55]
Isopropylparaben	+ ve (human MCF7) [54] + ve (yeast + receptor binding) [51]
Isobutylparaben	+ ve (human MCF7) [54] + ve (human MCF7; ZR-75-1) [40] + ve (yeast + receptor binding) [51]
Benzylparaben	+ ve (human MCF7; ZR-75-1) [43;50] + ve (yeast + receptor binding) [49-51] + ve (rat uterus receptor binding) [53]
***p*-hydroxybenzoic acid**	+ ve (human MCF7) [46]

5.4 SULT inhibition

Part of the effects of environmental estrogens is mediated via ERα and ERβ. However, recent studies have now shown that some xenestrogens may be able to exert endocrine disrupting properties through interfering with metabolic enzymes responsible for the synthesis of physiological estrogens or for modification of their availability in free, unconjugated form [56]. Prusakiewicz et al., 2007, report that parabens may also be able to inhibit sulfotransferases, which is another illustration of the different estrogen disrupting actions of parabens[25]. Estrogen action *in vivo* is regulated through a balanced interaction between sulfotransferase enzymes (SULTs) which catalyse sulfate conjugation and sulfatases which release free estrogens. Many of the environmental endocrine disrupters that disrupt the estrogen balance (e.g. hydroxylated polyhalogenated aromatic hydrocarbons) are compounds known to function as SULT inhibitors [57]. The finding that parabens can also inhibit sulfation of estrogens through inhibition of SULTs suggests that parabens may also indirectly enhance estrogen effects through elevation of free estradiol levels[25].

5.5 Antiandrogenic activity in vitro

Recent reports have documented that several parabens have the ability to bind to the androgen receptor and antiandrogenic activity was found for all the parabens tested [58;59]. Additionally, in a recent *in vitro* study, methyl-, propyl- and butyl-4-hydroxybenzoate were shown to be androgen receptor antagonists, and some of the parabens could inhibit testosterone induced transcriptional activity by as much as 40% at a concentration of 10 µM[59]. The inhibition of testosterone-induced transcription does not appear to correlate with side chain length, as methylparaben appears to be a more potent inhibitor than butyl- and propylparaben[59]. These observations provides a plausible and complementary explanation to the observed male reproductive disorders associated with parabens [26;27]. It is possible that some of these adverse effects are the result of lowered circulating androgen action by endocrine disrupting compounds which obstructs androgen signalling. The lack of a statistically significant inhibitory effect of PHBA in the study by Chen et al., 2007[59] indicates low, if any, antiandrogenic potency of this paraben metabolite. Table 5 below gives a summary of the published *in vitro* and *in vivo* studies on the antiandrogenic activity of parabens[2;27].

Table 5.*Antiandrogenic activity in vitro. Modified from [2].*	*AR binding assay*	*Reporter gene assay*	*Reference*
Methylparaben	− ve (recombinant hAR)	+ ve (transfected CHO-K1 cells) + ve (transfected HEK 293 cells)	[58] [59]
Ethylparaben	− ve (recombinant hAR)	+ ve (transfected CHO-K1 cells)	[58]
n-Propylparaben	+ ve (recombinant hAR)	+ ve (transfected CHO-K1 cells) + ve (transfected HEK 293 cells)	[58] [59]
n-Butylparaben	+ ve (recombinant hAR)	+ ve (transfected CHO-K1 cells) + ve (transfected HEK 293 cells)	[58] [59]
Isopropylparaben	+ ve (recombinant hAR)	+ ve (transfected CHO-K1 cells)	[58]
Isobutylparaben	+ ve (recombinant hAR	+ ve (transfected CHO-K1 cells)	[58]
p-Hydroxybenzoic acid		− ve (transfected HEK 293 cells)	[59]

Furthermore, Kjærstad et al. found that a mixture of five parabens exhibited pronounced AR antagonistic effects in a reporter gene assay, although only isobutylparaben was an antagonist on its own[60]. This may point to unexpected interactions between parabens, and further studies are needed to elucidate mixture effects of parabens *in vitro* and *in vivo*.

5.6 Mitochondrial toxicity of parabens

Tavares et al., 2009, reviewed the mitochondrial toxicity of parabens[61]. Mitochondria can be regarded as cell powerhouses producing the majority of the ATP used by cell processes. Mitochondrial toxicity of parabens have previously been reported by studies showing that parabens caused a concentration- and time-dependent cell death of cultured hepatocytes [62] with the effects correlated to defective mitochondrial function. This study also identified the mitochondrial respiratory chain and phosphorylation system as a target for the different parabens tested. It was demonstrated that butyl- and isobutylparaben were more toxic than propyl- and isopropylparaben, and ethyl- and methylparaben and *p*-hydroxybenzoic acid were less toxic than propylparaben, when considering mitochondria as the site for the toxic effect [62]. Later, it was pointed out that the mitochondrial permeability transition pore (MPT) is involved in the toxicity of different parabens in both hepatocytes and isolated liver mitochondria [63]. The opening of MPT pores increase the permeability of the mitochondrial membranes and can lead to mitochondrial swelling and cell death, and plays an important role in some types of apoptosis. The MPT is formed in the membranes of mitochondria under certain pathological conditions such as traumatic brain injury and stroke, and several agents are known to trigger MPT. For example, doxorubicin induces toxicity on testis mitochondria[64]. Studies on parabens as well as PHBA reported uncoupling and decrease of ATP synthesis associated with MPT induction[63]. Due to the important role of mitochondria in testis metabolism, Tavares *et al.*, (2009) conclude in their review that parabens may also interfere with mitochondrial energetics and thus disturb sperm function, although there are no data on paraben effects on testis mitochondria. Their preliminary results indicate that several parabens present direct toxicity on isolated testis mitochondria at low concentrations [61].

6 Blood levels and estrogenic effect concentrations

The present data on human blood levels of parabens and their metabolites are somewhat uncertain, but it is possible to estimate an estrogenic burden (estrogenic equivalency, EEQ) of parabens based on estrogenic effect factors (EEF) calculated from *in vitro* and *in vivo* data. In Table 6, blood levels of parent compounds and estradiol levels are compared and related to the relative potencies of these compounds. It should be noted, however, that also the estrogenic potency estimates contribute with considerable uncertainty to these calculations.

Table 6	*In vitro effect conc*	*Human blood concentration*	*EEF in vitro*	*EEQ = Conc * EEF in vitro (pM)*	*In vivo EEF (utero-trophic, CIR report)*	*EEQ = Conc * EEF in vivo (pM)*
Estradiol	10 pM		1		1	
- child, mean		5 pM (ref [65])		5		5
- child, range		1-50 pM (ref [65])		1-50		1-50
- woman		180 pM (ref [66])		180		180
- woman peak		1800 pM (ref [66])		1800		1800
- man		110 pM (ref [66])		110		110
Free ButP (dermal admin. of 800 mg ButP)	10^6 pM (ref [47])	$0.7*10^6$ pM (peak, ref [6])	10^{-5} (ref [47])	7	10^{-4}	70
		$0.1*10^6$ pM (24h, ref [6])		1		10
ProP (max levels in blood bank samples)		12800 pM (=2.3 ng/ml, ref [5])	10^{-5} (ref [47])	0.13	10^{-4}	1
PHBA (oral admin. of 600 mg EthP)	$500*10^6$ pM (ref [46])	$12*10^6$ pM (=2 µg/ml at peak, ref [7])	$0.5*10^{-6}$ (ref [44])	6	10^{-3} (10^{-4})	12000 (1200)
PHBA (estimated after dermal admin. of ButP)	$500*10^6$ pM (ref [44])	$8*10^6$ pM (at peak, ref [6]) ($=0.7*10^6$ pM*11)#	$0.5*10^{-6}$ (ref [44])	4	10^{-3} (10^{-4})	8000 (800)

EEQ: Estrogenic equivalency (pM)
EEF: Estrogenic effect factors
#: Estimated from a ratio of free paraben to PHBA 1:11 of, based on [7] in dogs. In human skin, more than 99% may be taken up as PHBA (ref [12]), and this estimate may be much larger).

The calculations in Table 6 reveal that the estrogenic capacity of peak levels of butylparaben applied to human test persons exceed the levels of estradiol in children. Free butyl paraben can be estimated to an EEQ of 70 pM whereas estradiol levels in children is approximately 5 pM (EEQ in vivo, ranging from 1 to 50 pM). Even 24 hours after dermal exposure [6], the estrogenic capacity of butylparaben (EEQ in vivo = 10 pM) is in the range of endogenous estradiol in children.

Even more remarkable are the propylparaben data, as the listed blood concentrations are measured in a background population. The maximal level of propylparaben leads to an estrogenic burden (EEQ in vivo=1 pM) equivalent to estradiol in children in some studies [65].

Heim et al., 1957 find a peak blood concentration of 12 uM of PHBA after administration of 600 mg ethylparaben[7]. This PHBA level is below the active concentration *in vitro*. However, calculation of estrogenic capacity based on *in vitro* potency reveals that the estrogenicity of PHBA (EEQ in vitro = 6 pM) is in the range of estradiol in children. Using uterotrophic potency data from Lemini et al., 2003[44], the estrogenic capacity of PHBA in serum exceeds serum estradiol levels in children more than 2000-fold (EEQ in vivo = 12000 pM). It should be noted, that this calculation is based on estrogenic equivalency factors estimated from a study showing effects of PHBA at 5 mg/kg bw/day, whereas other studies have shown that PHBA is inactive in the uterotrophic assay or is equally or less potent than the parabens (as discussed in section 5.2) [44]. Improved studies are needed to get more certainty on the estrogenic potency of parabens and PHBA.

Despite uncertainties in these calcutations, the comparisons in Table 6 indicate that even though human serum levels of intact parabens are low, the estrogenic capacity of the parent compounds may exceed the estradiol level in children – at least at peak serum levels of parabens. Normally, total dose levels or steady state levels of a given compound would be used for toxicity estimates. In this case, however, data are lacking and it is not known whether even exposures of short duration may be sufficient to induce an effect. The estrogenicity of PHBA may contribute significantly to this estrogenic load if the estrogenic potency of PHBA is indeed as high as implied in Table 6. Children may be particularly vulnerable to endocrine disruption by parabens, and other effects than their estrogen-like function must also be considered.

This is in contrast to the comparison of effective *in vitro* concentrations and human breast tumor levels by van Meeuwen et al., 2008. They conclude that parabens contribute marginally, if at all, to levels of circulating estrogens [47] (discussed in section 6.1). However, they consider paraben contribution to estrogen levels in adult women, and not the possibly more vulnerable children. Still, van Meeuwen et al., 2008, do express concern for the estrogenic burden of PHBA.

The PHBA level measured by Heim et al., 1957, is comparable to our estimate of serum PHBA level in humans exposed to butylparaben in the study by Janjua et al., 2007, although a large uncertainty applies to this estimate [6;6;7]. Further human studies are required to determine the true estrogenic equivalency of parabens and their metabolites. Additionally, the estrogenicity of PHBA differs between studies *in vivo* and *in vitro*. *In vitro* studies need to be supplied with metabolic studies in cells, as the “internal dose” in cells is unknown after exposure to chemicals in the media.

6.1 Are metabolites PHBA or PHHA responsible for endocrine disrupting effects of parabens?

In some, but not all, *in vitro* and *in vivo* studies, long chain parabens appear more toxic than short chain esters and PBHA (see section 5.2). It is possible that the difference in toxicity of long- and short-chain esters is related to differences in metabolism. Tsukamoto and Terada (1964) found that the urinary excretion of free PHBA was lower with longer chain lengths[10], and that the excretion of glycine-conjugated PHBA appeared slightly higher with increasing ester chain lengths. However, the metabolic differences between short-chain parabens and longer-chain esters do not appear to be as large as the toxicological differences of these compounds. However, the toxicity of methylparaben and ethylparaben may need to be revisited,

as also the SCCP opinion from 2006 remark that the study by Hoberman et al., 2008, may "undermine the decision taken earlier for methyl paraben" of an ADI of 10 mg/kg bw/day and that methylparaben can be safely used (up to a concentration of 0.4%).

PHBA is also a plant product with estimated human intake of PHBA up to 2-3 mg/day (0.04 mg/kg bw/day) from foods, mainly wine and berries [67]. The amount of PHBA from foods is therefore relatively small compared to the contribution of up to 1 mg/kg bw/day of PHBA from paraben exposure[4]. However, further studies may be required to compare exposure estimates of PHBA from various sources, as this may also be a metabolite of other compounds than those mentioned here.

The role of PHHA in the toxicity of parabens needs to be clarified by *in vitro* and *in vivo* investigations. Additionally, it needs to be determined whether subcutaneous or oral application of long-chain parabens leads to higher blood levels of pHHA than short-chain parabens. PHHA is also a marker of toluene exposure and may be found in pesticides, certain berries and grapes. PHHA is normally rapidly cleared, but in patients with renal failure, this compound accumulates in the blood and acts as a toxicant due to inhibition of the erythrocyte plasma membrane Ca^{2+}-ATPase[68]. To clarify the possible role of this metabolite, normal serum levels of PHHA needs to be determined and further comparison of sources of PHHA is needed.

According to studies in humans, less than 2% of unhydrolyzed paraben is present in a free form in urine, whereas the majority of paraben is present as a glucuronidated or sulphated conjugate. Although conjugated forms are assumed to be rapidly excreted it has not been clarified whether these conjugated parabens may have any potential endocrine disrupting effects.

7 Risk assessment

7.1 Exposure estimates

Cumulative exposure was estimated to 1.3 mg/kg bw/day based on refined aggregate exposure estimates in Cowan-Ellsbury et al., 2009[4]. Of this, 0.79, 0.34, and 0.0016 mg/kg bw/day was methyl-, propyl- and butylparaben, respectively. Using a more simple approach developed by SCCP in 2000 (then SCCFNP), Cowan-Ellsbury et al. calculated global exposure estimates for methylparaben, ethyl-, propyl-, and butylparaben of 1.0, 0.6, 0.8, and 0.02 mg/kg bw/day, respectively[4]. They concluded that the SCCP approach was a suitable, conservative screening tool for aggregate exposure estimates in personal care products.

Based on the study by Ye et al., 2006[9], an extrapolation of biomonitoring data to internal body exposure estimates was performed by Cowan-Ellsbury et al., 2009[4]. Cumulative internal exposure to parabens in a normal human population was estimated to 0.03 mg/kg bw/day, of which the majority was methylparaben. However, as no normalization of paraben data to creatinine clearance could be done in this study, Cowan-Ellsbury et al., 2009, estimated clearance based on phthalates as structural analogues of parabens. Therefore, better exposure estimates from biomonitoring data are needed and the more conservative aggregate exposure estimates listed above are used below.

7.2 Margin of safety

Current exposure data point to methyl- and propylparaben as the most abundant parabens with exposure levels up to 0.79 and 0.34 mg/kg bw/day, respectively. Cowan-Ellsbury et al., 2009, concluded that this was below an ADI of 10 mg/kg bw/day for all parabens[4]. However, this ADI refers to a JECFA paper from 1974, which obviously does not consider recent toxicity data. An EFSA expert panel (2004) established a group ADI of 0-10 mg/kg bw/day for methyl- and ethylparaben, but could not recommend an ADI for propylparaben (referred in [69]).

Considering a LOAEL of 10 mg/kg bw/day (based on Oishi, 2002[27]) and a factor of 3 for the lack of NOAEL (derived no-effect level of 3.3 mg/kg bw/day), the margin of safety for propylparaben is 3.3/0.34 = 10.

Considering a NOAEL of 1000 mg/kg bw/day (based on EU Scientific committee on Food, 1994), the margin of safety for methylparaben is 1000/0.79 = 1266.

However, this NOAEL of 1000 mg/kg bw/day for methylparaben does not consider the possible spermatotoxic effects in the study by Hoberman et al., 2008[28] as discussed in section 5. Furthermore, uterotrophic assays in immature mice have revealed effects at 20 mg/kg bw/day of propylparaben with a NOEL of 6.5 mg/kg bw/day [44]. In that study, methylparaben was uterotrophic from 16.5 mg/kg bw/day with a NOEL of 5.5 mg/kg bw/day. These values are very close to the maximal exposure estimates of 0.79 and 0.34 mg/kg bw/day estimated by Cowan-Ellsbury et al., 2009[4]. Using data for uterotrophic effects, margins of safety are 5.5/0.79 = 7 for methylparaben and 6.5/0.34 = 19 for propylparaben (Table 7).

Table 7. Safety evaluation based on aggregate exposure estimates	Exposure estimate[4] (mg/kg bw/day)	LOAEL/NOAEL (mg/kg bw/day)	Margin of safety
Methyl paraben	0.8	1000 (male effects) 5.5 (uterotrophic[44])	1266 7
Propyl paraben	0.3	10 (LOAEL, male effects[27]) 6.5 (uterotrophic[44])	10 19
Butyl paraben	0.0016	10 (LOAEL, male effects[26]) 0.7 (uterotrophic[44])	2083 437

7.3 Estrogenic burden of parabens in children

The estrogenic capacity of parabens calculated in table 6 indicates that free, intact parabens may be present in concentrations with estrogenic effects comparable to endogenous estrogens. This may in itself be a cause of concern, and when considering the possible contribution of metabolites to this estrogenic burden, the potential estrogenic influence of paraben exposure in childhood seems alarmingly high. From this perspective, not only the long-chain parabens may be of concern, but also the short-chain esters and PHBA.

Parabens affect reproductive or endocrine endpoints in both male and female immature rats and mice, and with human exposure both boys and girls may be at risk of endocrine disruption. Estrogenic effects in boys may increase the risk for incomplete masculinization resulting in decreased sperm quality. In girls, an increased estrogenic load may increase the risk of early puberty, premature mammary development and the risk of mammary cancer. However, the estrogenicity of parabens and their metabolites needs to be compared to the possible risk of exposure to other sources of estrogens (e.g. phytoestrogens).

8 Research needs

According to the home page for the National Toxicology Programme (NTP), US, butylparaben is "on test" in an ADME study in rats. Gavage, intravenous and topical application is listed as routes of exposure for the ADME study [70]. Additionally, butylparaben is selected for testing in a continuous breeding programme at the NTP. The box below is a copy of the Concept Document developed by the NTP prior to initiating the testing.

Development by a study team of detailed proposals was recommended to incorporate the following toxicological issues in priority order:
1. To undertake *in vitro* / high throughput studies aimed at a number of potential toxicities of BP, but especially (i) interaction with steroid receptors (eg Androgen Receptor) (ii) evaluation of effects on steroidogenesis (eg in H295R cells) (iii) mitochondrial function. It was recommended that such studies should not only include BP and its likely major metabolites (eg p-hydroxybenzoic acid), but other members of the parabens class (and their presumed metabolites).
2. To undertake a series of directed TK studies in the SD rat to compare oral with dermal exposure. This would involve blood levels of parent compound and metabolites, especially those found "active" from the *in vitro* studies in 1. Studies should also incorporate exposure during pregnancy to evaluate fetal levels of BP and metabolites. Dual labeling of the ring and side chain was suggested as a possibility to evaluate the stability of the ester side chain.
3. To conduct a modified RACB study in the SD rat to evaluate functional effects on reproduction and post-natal development. The design of this study should incorporate sufficient additional components so as to provide an appropriate sub-chronic evaluation of the parental generation (including hematology, detailed pathology etc) to substitute for a stand-alone 90-day rat study. The use of the SD rat should be fully justified based on its preference for use in the RACB, over the standard, F/344 rat (i.e. historical control data available). The F344 has a smaller litter size, poor maternal behavior and is specifically not recommend for reproduction studies in standard regulatory reproductive toxicity guidelines. If possible, the study team should evaluate whether some F1 animals may be held for at least 90 days after birth to evaluate potential testicular neoplasia (similar to that previously noted with phthalates).

In line with this, we suggest improved toxicokinetic studies and toxicity studies in vitro and in vivo as listed below.

8.1 Experimental animals

- Improved studies on absorbtion, distribution, metabolism and elimination (ADME) are required. When conducting studies on administration of radioactively labeled parabens it is important to evaluate the recovery of metabolites to determine which metabolites are not readily excreted in the urine. These studies should include detection of labeled metabolites in organs. It is possible that this information can be obtained from the studies recently submitted by COLIPA to the SCCP, but these data have not been made available to us.

- Studies should include the intravenous route in order to calculate bioavailability of compounds (bioavailability F = AUCdermal / AUCiv if doses are similar).

- Organ levels should be compared to effective concentrations *in vitro*, as there appears to be some degree of up concentration of parabens in fetuses and organs compared to blood [17].

- An extended one generation study or a two generation study on methyl-, ethyl-, butyl-, propylparaben and isoforms could is proposed.

- Rat studies should include lower levels of propylparaben with oral or subcutaneous dosing. No studies on effects of subcutaneous exposure of young males have been reported, but only dietary studies. Subcutaneous exposure is relevant for paraben studies, as the first-pass effect (metabolism in liver) is avoided similarly to human exposure to parabens via dermal application. Uterotrophic studies in immature mice show effects at low doses with subcutaneous administration. Propylparaben has a LOAEL of 10 mg/kg bw/day for male effects based on the study by Oishi, 2002[27], and human exposure is estimated to up to 0.3 mg/kg bw/day indicating a low margin of safety. Consequently, it is important to improve the knowledge on the no-effect level for propylparaben.

- The possible toxicity of paraben hydrolysis products PHHA and PHBA should be investigated further. It may also be relevant to determine whether subcutaneous or oral application of long-chain parabens leads to higher blood levels of pHHA than short-chain parabens. The possible toxicity of the glucuronidated and sulphated forms of unhydrolyzed parabens may also need investigation.

- Combination studies should be performed to investigate whether the combined exposure to several parabens or to parabens together with other endocrine disrupting compounds leads to cumulative effects.

- Studies on other estrogenic chemicals of varying estrogenic potency have shown effects on mammary gland development at low doses. Changes in early mammary development may affect lactation capacity and affect the vulnerability to breast cancer later in life. Parabens have been suspected of being causally related to breast cancer due to their estrogenic effect[2]. To our knowledge, no studies of paraben influence on mammary development in rodents have been performed in vivo.

8.2 Human studies

- Improved studies on uptake and metabolism in exposed humans as well as background populations are needed. Data should determine uptake of a) free parabens, b) conjugated parent compounds, c) PHBA, D) conjugated metabolites of PHBA including PHHA.

- As mentioned above for the animal studies, it is important to evaluate the recovery of metabolites to determine which other metabolites than those already described may be formed.

8.3 *In vitro* studies

- Cellular metabolism of compounds should be studied *in vitro* in order to determine if metabolites are active. Additionally, other metabolites than those previously described (including phase 1 metabolites) may be identified.

- In order to elaborate on metabolite effects, p-hydroxy hippuric acid should be studied further, and *in vitro* estrogenic effects of PHBA should be confirmed.

- Combination studies are required to elaborate further on the cumulative effects parabens *in vitro* as observed in AR reporter gene assays[60]. As humans are likely to be exposed to several parabens together with other chemicals with estrogenic, anti-androgenic or other effects, the interactions between parabens and other compounds is also important.

9 Conclusions

- After dermal uptake, parabens are hydrolyzed and conjugated and excreted in urine. Total dermal uptake of paraben and metabolites is quite high, and has been estimated to be around 50%; maybe up to 80% of exposure. Further studies are needed to determine which metabolites are present in blood and organs and at which levels.

- Only a small amount of intact parabens can be recovered in human blood and urine, and it can be estimated that around 2% of the applied dose is excreted in urine in an intact form (conjugated and free).

- Species differences between humans and experimental animals point to higher uptake and less metabolism in human skin than in the applied rat models. This would lead to higher internal doses in exposed humans than rats.

- The ability of parabens to activate the estrogen receptor may not be the main mechanism of action, as they also show androgen receptor antagonism, ability to elevate estrogen levels via SULT inhibition, and mitochondrial toxicity.

- Estrogenic burden of free parabens and PHBA in blood may exceed estradiol levels in children. However, estimates on estrogenic potencies as well as blood concentrations of parabens and PHBA are somewhat uncertain.

- Margin of safety for propyl paraben is very low when comparing worst-case exposure data to NOAELs from studies on young male rats or uterotrophic studies (immature mice).

- Additional studies are needed, in particular reproduction studies on both long- and short-chain parabens, as well as extended ADME studies, combination studies in vitro and in vivo and studies of novel endpoints such as mammary development.

References

(1) Final amended report on the safety assessment of Methylparaben, Ethylparaben, Propylparaben, Isopropylparaben, Butylparaben, Isobutylparaben, and Benzylparaben as used in cosmetic products. Int J Toxicol 2008; 27 Suppl 4:1-82.

(2) Darbre PD, Harvey PW. Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. J Appl Toxicol 2008; 28(5):561-578.

(3) Scientific Committee on Consumer Products (SCCP). Extended opinion on the safety evaluation of parabens. SCCP/0873/05. 2005.

(4) Cowan-Ellsberry CE, Robison SH. Refining Aggregate Exposure: Example using Parabens. Regul Toxicol Pharmacol 2009.

(5) Ye X, Tao LJ, Needham LL, Calafat AM. Automated on-line column-switching HPLC-MS/MS method for measuring environmental phenols and parabens in serum. Talanta 2008; 76(4):865-871.

(6) Janjua NR, Mortensen GK, Andersson AM, Kongshoj B, Skakkebaek NE, Wulf HC. Systemic uptake of diethyl phthalate, dibutyl phthalate, and butyl paraben following whole-body topical application and reproductive and thyroid hormone levels in humans. Environ Sci Technol 2007; 41(15):5564-5570.

(7) HEIM F, LEUSCHNER F, WUNDERLICH G. [Intermediary behavior of p-hydroxybenzoic acid ethyl ester.]. Klin Wochenschr 1957; 35(16):823-825.

(8) Janjua NR, Frederiksen H, Skakkebaek NE, Wulf HC, Andersson AM. Urinary excretion of phthalates and paraben after repeated whole-body topical application in humans. Int J Androl 2008; 31(2):118-130.

(9) Ye X, Bishop AM, Reidy JA, Needham LL, Calafat AM. Parabens as urinary biomarkers of exposure in humans. Environ Health Perspect 2006; 114(12):1843-1846.

(10) TSUKAMOTO H, TERADA S. METABOLISM OF DRUGS. XLVII. METABOLIC FATE OF RHO-HYDROXYBENZOIC ACID AND ITS DERIVATIVES IN RABBIT. Chem Pharm Bull (Tokyo) 1964; 12:765-769.

(11) Yamashita F, Bando H, Koyama Y, Kitagawa S, Takakura Y, Hashida M. In vivo and in vitro analysis of skin penetration enhancement based on a two-layer diffusion model with polar and nonpolar routes in the stratum corneum. Pharm Res 1994; 11(2):185-191.

(12) Fasano WJ. Butylparaben: in vitro kinetics and metabolism using full thickness human skin. E.I. du Pont de Nemours and Company. Haskell Laboratory (Dupont-14795). Unpublished data. Sponsor: Cosmetic, Toiletry, and Fragance Association (CFTA). 2005.

(13) Jewell C, Prusakiewicz JJ, Ackermann C, Payne NA, Fate G, Voorman R, Williams FM. Hydrolysis of a series of parabens by skin microsomes and cytosol from human and minipigs and in whole skin in short-term culture. Toxicol Appl Pharmacol 2007; 225(2):221-228.

(14) Seko N, Bando H, Lim CW, Yamashita F, Hashida M. Theoretical analysis of the effect of cutaneous metabolism on skin permeation of parabens based on a two-layer skin diffusion/metabolism model. Biol Pharm Bull 1999; 22(3):281-287.

(15) Bando H, Mohri S, Yamashita F, Takakura Y, Hashida M. Effects of skin metabolism on percutaneous penetration of lipophilic drugs. J Pharm Sci 1997; 86(6):759-761.

(16) Pedersen S, Marra F, Nicoli S, Santi P. In vitro skin permeation and retention of parabens from cosmetic formulations. Int J Cosmet Sci 2007; 29(5):361-367.

(17) Frederiksen H, Taxvig C, Hass U, Vinggaard AM, Nellemann C. Higher levels of ethyl paraben and butyl paraben in rat amniotic fluid than in maternal plasma after subcutaneous administration. Toxicol Sci 2008; 106(2):376-383.

(18) Nohynek,G., European Cosmetics Association, presentation at meeting at Danish Environmental Protection Agency with The Association of Danish Cosmetics and Detergent Industries, August 19th 2009. 2009.

(19) Kiwada H, Awazu S, Hanano M. The study on the biological fate of paraben at the dose of practical usage in rat. II. The pharmacokinetic study on the blood concentration after the administration of ethyl paraben or p-hydroxybenzoic acid. J Pharmacobiodyn 1980; 3(7):353-363.

(20) Nohynek,G., European Cosmetics Association, presentation at meeting at Danish Environmental Protection Agency with The Association of Danish Cosmetics and Detergent Industries, August 19th 2009. 2009.

(21) Scientific Committee on Consumer Products (SCCP) Opinion on parabens. SCCP/1017/06. 2006.

(22) Harville HM, Voorman R, Prusakiewicz JJ. Comparison of paraben stability in human and rat skin. Drug Metab Lett 2007; 1(1):17-21.

(23) Williams FM. Potential for metabolism locally in the skin of dermally absorbed compounds. Hum Exp Toxicol 2008; 27(4):277-280.

(24) Jewell C, Bennett P, Mutch E, Ackermann C, Williams FM. Inter-individual variability in esterases in human liver. Biochem Pharmacol 2007; 74(6):932-939.

(25) Prusakiewicz JJ, Ackermann C, Voorman R. Comparison of skin esterase activities from different species. Pharm Res 2006; 23(7):1517-1524.

(26) Oishi S. Effects of butylparaben on the male reproductive system in rats. Toxicol Ind Health 2001; 17(1):31-39.

(27) Oishi S. Effects of propyl paraben on the male reproductive system. Food Chem Toxicol 2002; 40(12):1807-1813.

(28) Hoberman AM, Schreur DK, Leazer T, Daston GP, Carthew P, Re T, Loretz L, Mann P. Lack of effect of butylparaben and methylparaben on the reproductive system in male rats. Birth Defects Res B Dev Reprod Toxicol 2008; 83(2):123-133.

(29) Ruhlen RL, Howdeshell KL, Mao J, Taylor JA, Bronson FH, Newbold RR, Welshons WV, vom Saal FS. Low phytoestrogen levels in feed increase fetal serum estradiol resulting in the "fetal estrogenization syndrome" and obesity in CD-1 mice. Environ Health Perspect 2008; 116(3):322-328.

(30) Herath CB, Jin W, Watanabe G, Arai K, Suzuki AK, Taya K. Adverse effects of environmental toxicants, octylphenol and bisphenol A, on male reproductive functions in pubertal rats. Endocrine 2004; 25(2):163-172.

(31) Oishi S. Lack of spermatotoxic effects of methyl and ethyl esters of p-hydroxybenzoic acid in rats. Food Chem Toxicol 2004 Nov ;42 (11):1845 -9 2004; 42:1845-1849.

(32) Kang KS, Che JH, Ryu DY, Kim TW, Li GX, Lee YS. Decreased sperm number and motile activity on the F1 offspring maternally exposed to butyl p-hydroxybenzoic acid (butyl paraben). J Vet Med Sci 2002; 64(3):227-235.

(33) Taxvig C, Vinggaard AM, Hass U, Axelstad M, Boberg J, Hansen PR, Frederiksen H, Nellemann C. Do parabens have the ability to interfere with steroidogenesis? Toxicol Sci 2008; 106(1):206-213.

(34) Daston GP. Developmental toxicity evaluation of butylparaben in Sprague-Dawley rats. Birth Defects Res B Dev Reprod Toxicol 2004; 71(4):296-302.

(35) Lemini C, Silva G, Timossi C, Luque D, Valverde A, Gonzalez-Martinez M, Hernandez A, Rubio-Poo C, Chavez LB, Valenzuela F. Estrogenic effects of p-hydroxybenzoic acid in CD1 mice. Environ Res 1997; 75(2):130-134.

(36) Hossaini A, Larsen JJ, Larsen JC. Lack of oestrogenic effects of food preservatives (parabens) in uterotrophic assays. Food Chem Toxicol 2000; 38(4):319-323.

(37) Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. Toxicol Appl Pharmacol 1998; 153(1):12-19.

(38) Lemini C, Hernandez A, Jaimez R, Franco Y, Avila ME, Castell A. Morphometric analysis of mice uteri treated with the preservatives methyl, ethyl, propyl, and butylparaben. Toxicol Ind Health 2004; 20(6-10):123-132.

(39) Shaw J, deCatanzaro D. Estrogenicity of parabens revisited: impact of parabens on early pregnancy and an uterotrophic assay in mice. Reprod Toxicol 2009; 28(1):26-31.

(40) Darbre PD, Byford JR, Shaw LE, Horton RA, Pope GS, Sauer MJ. Oestrogenic activity of isobutylparaben in vitro and in vivo. J Appl Toxicol 2002; 22(4):219-226.

(41) Koda T, Umezu T, Kamata R, Morohoshi K, Ohta T, Morita M. Uterotrophic effects of benzophenone derivatives and a p-hydroxybenzoate used in ultraviolet screens. Environ Res 2005; 98(1):40-45.

(42) Vo TT, Jeung EB. An evaluation of estrogenic activity of parabens using uterine calbindin-D9k gene in an immature rat model. Toxicol Sci 2009.

(43) Darbre PD, Byford JR, Shaw LE, Hall S, Coldham NG, Pope GS, Sauer MJ. Oestrogenic activity of benzylparaben. J Appl Toxicol 2003; 23(1):43-51.

(44) Lemini C, Jaimez R, Avila ME, Franco Y, Larrea F, Lemus AE. In vivo and in vitro estrogen bioactivities of alkyl parabens. Toxicol Ind Health 2003; 19(2-6):69-79.

(45) Byford JR, Shaw LE, Drew MG, Pope GS, Sauer MJ, Darbre PD. Oestrogenic activity of parabens in MCF7 human breast cancer cells. J Steroid Biochem Mol Biol 2002; 80(1):49-60.

(46) Pugazhendhi D, Pope GS, Darbre PD. Oestrogenic activity of p-hydroxybenzoic acid (common metabolite of paraben esters) and methylparaben in human breast cancer cell lines. J Appl Toxicol 2005; 25(4):301-309.

(47) van Meeuwen JA, van SO, Piersma AH, de Jong PC, van den BM. Aromatase inhibiting and combined estrogenic effects of parabens and estrogenic effects of other additives in cosmetics. Toxicol Appl Pharmacol 2008; 230(3):372-382.

(48) Gee RH, Charles A, Taylor N, Darbre PD. Oestrogenic and androgenic activity of triclosan in breast cancer cells. J Appl Toxicol 2008; 28(1):78-91.

(49) Miller D, Wheals BB, Beresford N, Sumpter JP. Estrogenic activity of phenolic additives determined by an in vitro yeast bioassay. Environ Health Perspect 2001; 109(2):133-138.

(50) Schultis T, Metzger JW. Determination of estrogenic activity by LYES-assay (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase). Chemosphere 2004; 57(11):1649-1655.

(51) Morohoshi K, Yamamoto H, Kamata R, Shiraishi F, Koda T, Morita M. Estrogenic activity of 37 components of commercial sunscreen lotions evaluated by in vitro assays. Toxicol In Vitro 2005; 19(4):457-469.

(52) Vanparys C, Maras M, Lenjou M, Robbens J, Van BD, Blust R, De CW. Flow cytometric cell cycle analysis allows for rapid screening of estrogenicity in MCF-7 breast cancer cells. Toxicol In Vitro 2006; 20(7):1238-1248.

(53) Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 2000; 54(1):138-153.

(54) Okubo T, Yokoyama Y, Kano K, Kano I. ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ERalpha and PR. Food Chem Toxicol 2001; 39(12):1225-1232.

(55) Gomez E, Pillon A, Fenet H, Rosain D, Duchesne MJ, Nicolas JC, Balaguer P, Casellas C. Estrogenic activity of cosmetic components in reporter cell lines: parabens, UV screens, and musks. J Toxicol Environ Health A 2005; 68(4):239-251.

(56) Whitehead SA, Rice S. Endocrine-disrupting chemicals as modulators of sex steroid synthesis. Best Pract Res Clin Endocrinol Metab 2006; 20(1):45-61.

(57) Kester MH, Bulduk S, van Toor H, Tibboel D, Meinl W, Glatt H, Falany CN, Coughtrie MW, Schuur AG, Brouwer A, Visser TJ. Potent inhibition of estrogen sulfotransferase by hydroxylated

metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. J Clin Endocrinol Metab 2002; 87(3):1142-1150.

(58) Satoh K, Nonaka R, Ohyama K, Nagai F. Androgenic and antiandrogenic effects of alkylphenols and parabens assessed using the reporter gene assay with stably transfected CHO-K1 cells (AR-EcoScreen System). J Health Sci 2005; 51(5):557-568.

(59) Chen J, Ahn KC, Gee NA, Gee SJ, Hammock BD, Lasley BL. Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products. Toxicol Appl Pharmacol 2007; 221(3):278-284.

(60) Kjaerstad MB, Taxvig C, Andersen HR, Nellemann C. Mixture effects of endocrine disrupting compounds in vitro (Submitted). Int J Androl 2009.

(61) Tavares RS, Martins FC, Oliveira PJ, Ramalho-Santos J, Peixoto FP. Parabens in male infertility-is there a mitochondrial connection? Reprod Toxicol 2009; 27(1):1-7.

(62) Nakagawa Y, Moldeus P. Mechanism of p-hydroxybenzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat hepatocytes. Biochem Pharmacol 1998; 55(11):1907-1914.

(63) Nakagawa Y, Moore G. Role of mitochondrial membrane permeability transition in p-hydroxybenzoate ester-induced cytotoxicity in rat hepatocytes. Biochem Pharmacol 1999; 58(5):811-816.

(64) Yeh YC, Lai HC, Ting CT, Lee WL, Wang LC, Wang KY, Lai HC, Liu TJ. Protection by doxycycline against doxorubicin-induced oxidative stress and apoptosis in mouse testes. Biochem Pharmacol 2007; 74(7):969-980.

(65) Bay K, Andersson AM, Skakkebaek NE. Estradiol levels in prepubertal boys and girls--analytical challenges. Int J Androl 2004; 27(5):266-273.

(66) Berne RM, Levy MN. Physiology. 4 ed. St Louis: Mosby; 1998.

(67) Radtke J, Linseisen J, Wolfram G. [Phenolic acid intake of adults in a Bavarian subgroup of the national food consumption survey]. Z Ernahrungswiss 1998; 37(2):190-197.

(68) Jankowski J, Tepel M, Stephan N, van der GM, Breden V, Zidek W, Schluter H. Characterization of p-hydroxy-hippuric acid as an inhibitor of Ca2+-ATPase in end-stage renal failure. Kidney Int Suppl 2001; 78:S84-S88.

(69) Soni MG, Burdock GA, Taylor SL, Greenberg NA. Safety assessment of propyl paraben: a review of the published literature. Food Chem Toxicol 2001; 39(6):513-532.

(70) http://ntp.niehs.nih.gov/index.cfm?objectid=BD7AD768-123F-7908-7B0342F6AF18B69D. 2009.