A Proposed Study on the Transplacental Transport of Parabens in the Human Placental Perfusion Model

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Summary — Human exposure to parabens as a preservative used in personal care products is of increasing concern, as there is evidence from *in vivo* and *in vitro* studies of hormone disruption in association with exposure to parabens. Transport across the placenta could be critical for risk assessment, but the available data are sparse. The aim is to develop a method for estimating fetal exposure, via the placenta, to the most commonly-used parabens, by using a human placental perfusion model. The use of human tissue is vital for determining human fetal exposure, because animal studies are of little relevance, since the placenta exhibits significant interspecies variation. An HPLC model is currently being established to simultaneously quantify four different parabens, namely, methylparaben, ethylparaben, propylparaben and butylparaben, and their main metabolite, *p*-hydroxybenzoic acid. With this model, we aim to determine the transport kinetics of these parabens across the human placenta, and to investigate placental metabolism, including differences in transport due to molecular characteristics. This will facilitate assessment of the risks associated with the use of paraben-containing products during pregnancy.

Key words: *cosmetics,* ex vivo*, fetal exposure, HPLC, human transplacental transport, parabens,* p-*hydroxybenzoic acid, placental perfusion, preservative.*

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Introduction

There has been increasing concern about the safety of the parabens, used individually or in mixtures, as preservatives in a wide variety of cosmetics and healthcare products, as well as in foodstuffs and pharmaceuticals (1, 2). However, there have been relatively few studies, and the available data make it difficult to make rational risk assessments.

Parabens

Parabens are derivatives of 4-hydroxybenzoic acid, esterified at the *para* position (3). The commonly-used parabens include those with an alkyl group ranging from C_1 to C_4, i.e. methyl paraben (MeP), ethyl paraben (EtP), propyl paraben (PrP) and butyl paraben (BuP) (see Table 1). They are used because of their bactericidal and fungicidal properties, and this, together with their low cost and low sensitising potential, has resulted in their widespread use, either individually or in mixtures, as antimicrobial preservatives in cosmetics, foodstuffs and drugs (1, 2). All the parabens used in commercial products are synthetically produced, but parabens also occur naturally in plant-derived products intended for consumption, including fruit juice and white wine (4–6). In plants, parabens have been shown to exert an antimicrobial action (6).

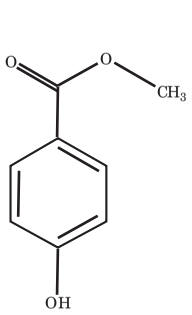
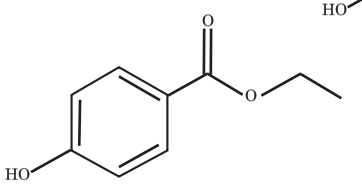
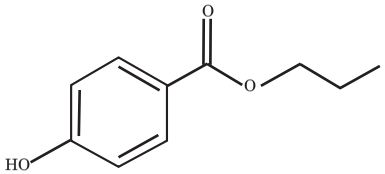
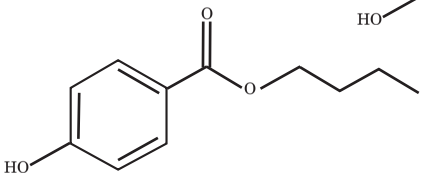
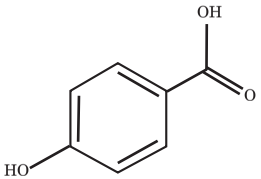
Exposure to parabens

Human exposure to parabens occurs through ingestion, absorption through the skin, and inhalation (7, 8). Transdermal absorption is related to the ability of parabens to cross the stratum corneum, which, in turn, is linked to their alkyl chain lengths (9). Other factors, including the inherent chemical properties of parabens, their metabolism (by hydrolysis of the ester bond and by glucoronidation), and the formulations of the cosmetic end-products in which these preservatives are used (which include shampoos, conditioners, other hair products, sunscreens, moisturisers, mascaras, and shaving products), also affect the absorption of parabens (10, 11). Due to the large areas of skin to which products could be applied and their use on a daily basis, even a low level of penetration could result in accumulation in the body tissues (12).

The levels of exposure to parabens from various sources have been assessed, to provide an estimated daily intake (EDI; 13), which is 1.01mg/kg

[a]*This author was a 2012 Lush Young Researcher Prize winner.*

Table 1: General information and physicochemical properties regarding the four most common parabens and their main metabolites

General information					
Structural formula					
Molecular formula	$C_8H_8O_3$	$C_9H_{10}O_3$	$C_{10}H_{12}O_3$	$C_{11}H_{14}O_3$	$C_7H_6O_3$
Name	Methyl paraben	Ethyl paraben	Propyl paraben	Butyl paraben	*p*-hydroxybenzoic acid
MW (g/mol)	152.15	166.17	180.20	194.23	138.121
CAS No.	99-76-3	120-47-8	94-13-3	94-26-8	99-96-7
Physicochemical properties					
Log K_{ow}	1.96	2.47	3.04	3.57	1.58
pK_a	7.91	8.34	7.91	8.47	4.54
S[a](mg/L)	2×10^3	9×10^2	5×10^2	2×10^2	5×10^3
Health Effects					
Dermatitis exacerbation, skin irritation and rosacea in subjects with pre-existing allergy to parabens	Yes	Yes	Yes	Yes	—
Oxidative stress potential	Yes	Yes	No	No	—
DNA damage	No	No	No	Yes	—

The information was obtained from References 4, 8, 14, 24 and 48. [a]Solubility in water at 25°C. — = no information available.

body weight/day for men and 1.06mg/kg body weight/day for women (Figure 1; 1), of which the intake from pharmaceuticals and cosmetics is estimated to be 0.42 and 0.83mg/kg/day, respectively (4). Nevertheless, additional studies should be carried out, in order to investigate new environmental sources of exposure to parabens (1).

Several studies have been used to determine the concentration of MeP, EtP, PrP and BuP in the urine of men and pregnant women, but there is still a lack of data regarding the concentration of parabens in various body tissues (Tables 2 and 3). In addition, the potential for parabens to accumulate in the body as a result of the daily application of several different parabens-containing products, needs to be investigated (14). Further studies might also be significant for improving the regulation of the use of parabens.

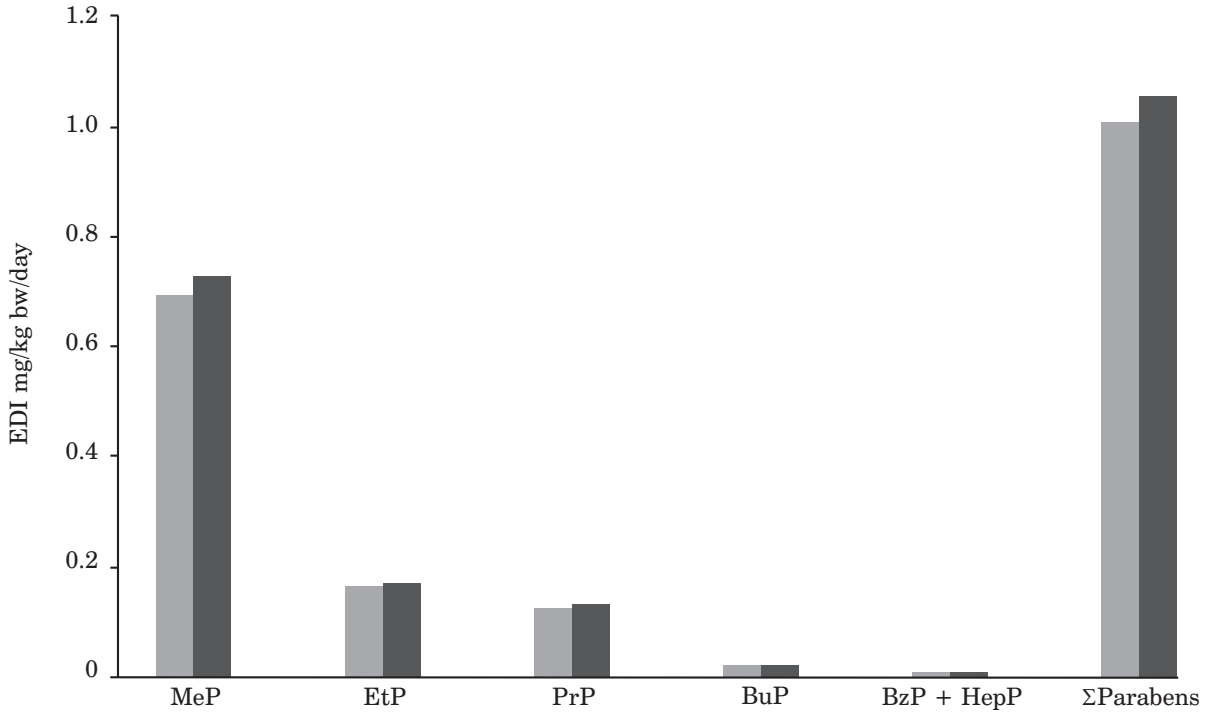
In the EU, the use of MeP, EtP and PrP as preservatives in foods is regulated by EU *Directive 64/54/EEC*, and the use of parabens in cosmetic products is permitted according to *Directive 76/768/EEC* (15) and its various adaptations to technical progress, up to a maximum concentration of 0.4% v/v when used singularly and 0.8% when used as a mixture (14). A scientific committee at the European Food Safety Authority (EFSA) has established the Acceptable Daily Intake (ADI) of 0–10mg/kg/day for the combined sum of MeP and EtP (16). It was not possible to establish an ADI for PrP, as *in vivo* studies had highlighted its possible effects as an oestrogen-like substance (4).

The effects of parabens

Several studies involving the use of animal models have been carried out, in order to assess the adverse effects of parabens (17, 18). Acute oral toxicity and lethal doses for parabens used singularly in dogs and rabbits ranged from 2 to 6g/kg body weight (17, 18), which was higher than 2g/kg body weight for acute dermal toxicity in rats (2). However, these doses are 2,000 to 6,000 times higher than the EDI for humans, thus making these exposures unrealistic and irrelevant (1). In any case, the extrapolation of data from animals to humans is subject to error, due to interspecies differences.

A number of studies on the adverse effects of human exposure to parabens have provided some evidence of their sensitisation potential, oestrogen-like activity, and oxidative potential (8, 19–21).

Figure 1: The estimated daily intake of parabens from foodstuff



= *Chinese male subjects;* = *Chinese female subjects.*

The bar chart was created by using data from a study performed in China (1) to determine the estimated daily intake (EDI) of parabens from foodstuff, in mg/kg bw/day. BzP = benzyl paraben; HepP = heptyl paraben; others as defined in main text.

Table 2: Reported parabens levels in the urine of pregnant women

Country	*n*	Median urinary paraben concentration (μg/L)			
		MeP	**EtP**	**PrP**	**BuP**
Korea	46	134 (31.7–475.0) **98%**	38.0 (9.9–235.0) **100%**	6.6 (0.37–55.2) **98%**	ND (ND–0.67) **28%**
Japan	111	75.8 (27.4–164.0) **94%**	7.53 (1.37–25.8) **81%**	20.2 (7.73–84.6) **89%**	0.59 (<0.46–3.34) **54%**
Spain	120	191 (415.5) **100%**	8.8 (25.7) **88%**	29.8 (61.3) **98%**	2.4 (10.3) **90%**
France	191	97.8 (9.1–3520) **100%**	4.1 (0.7–62.3) **68%**	12.5 (0.5–402.0) **97%**	1.7 (0.1–53.8) **80%**
USA	129	135 (51.3–287) —	—	22.8 (7.33–75.2) —	0.88 (0.25–8.88) —

The interquartile range is shown in brackets and the percentage detection is indicated in bold. The information was obtained from References 16, 19 and 49–52. — = no information available; ND = not determined.

For example, MeP, EtP, PrP and BuP were tested on 50 humans, at concentrations ranging from 5 to 15% paraben in propylene glycol (22). The test was conducted over five days, with daily application of the chemicals on the backs of the participants. MeP and BuP gave the highest sensitisation potential — the lowest concentration of these parabens that did not elicit irritation was 5%, whereas the respective values for EtP and PrP were 7% and 12%. In addition, no photosensitisation or phototoxicity were observed during the experiment. This was consistent with the results obtained in another study, on male subjects aged 21 to 50, each of whom had an occlusive patch containing a mixture of MeP and PrP applied to an arm for 3.5 weeks. This exposure period was followed by a two-week period without exposure to the parabens, then a new application was carried out to assess whether sensitisation had occurred (23).

However, the widespread use of parabens means that sensitisation to them may have already occurred before exposure to a particular product was considered. As a result, individuals with a pre-existing allergy to parabens may suffer from skin irritation, dermatitis or rosacea (24). For this reason, the addition of PrP and BuP to products intended for use by children has been prohibited in Denmark, since, for example, their application to damaged skin in the nappy area of children below 6 months, might elicit sensitisation. Nevertheless, no formal risk assess-

Table 3: Reported parabens levels in male urine

Country	*n*	Median urinary paraben concentration (μg/L)			
		MeP	**EtP**	**PrP**	**BuP**
Denmark	60	17.7 (6.58–64.6) **98%**	1.98 (0.49–5.35) **100%**	3.60 (0.85–14.0) **98%**	0.19 (0.09–1.01) **28%**
USA	194	27.4 (11.4–64.8) **100%**	—	3.45 (0.80–17.1) **92%**	ND (ND–0.30) **54%**

The interquartile range is shown in brackets and the percentage detection is indicated in bold. The information was obtained from References 8, 52 and 53. — = no information available; ND = not determined.

ment concerning parabens is currently available for this group in the population (25).

Concerns have also been raised about the possible adverse effects of the oestrogenic, androgenic, anti-oestrogenic or anti-androgenic properties of parabens. Such adverse effects could affect the reproductive system, and effects on the fetus could occur during pregnancy or in later life. For example, the *in utero* exposure of females could lead to the early onset of puberty, and the anti-androgenic effects of chemicals on the male fetus could contribute to a reduction in male reproductive capability (26). Denmark is characterised by a very low reproductive success-rate compared to other European countries. Elucidating the cause of this phenomenon is of great importance, and to this end, the Danish Environmental Protection Agency has financed a centre for research on endocrine disruption (27).

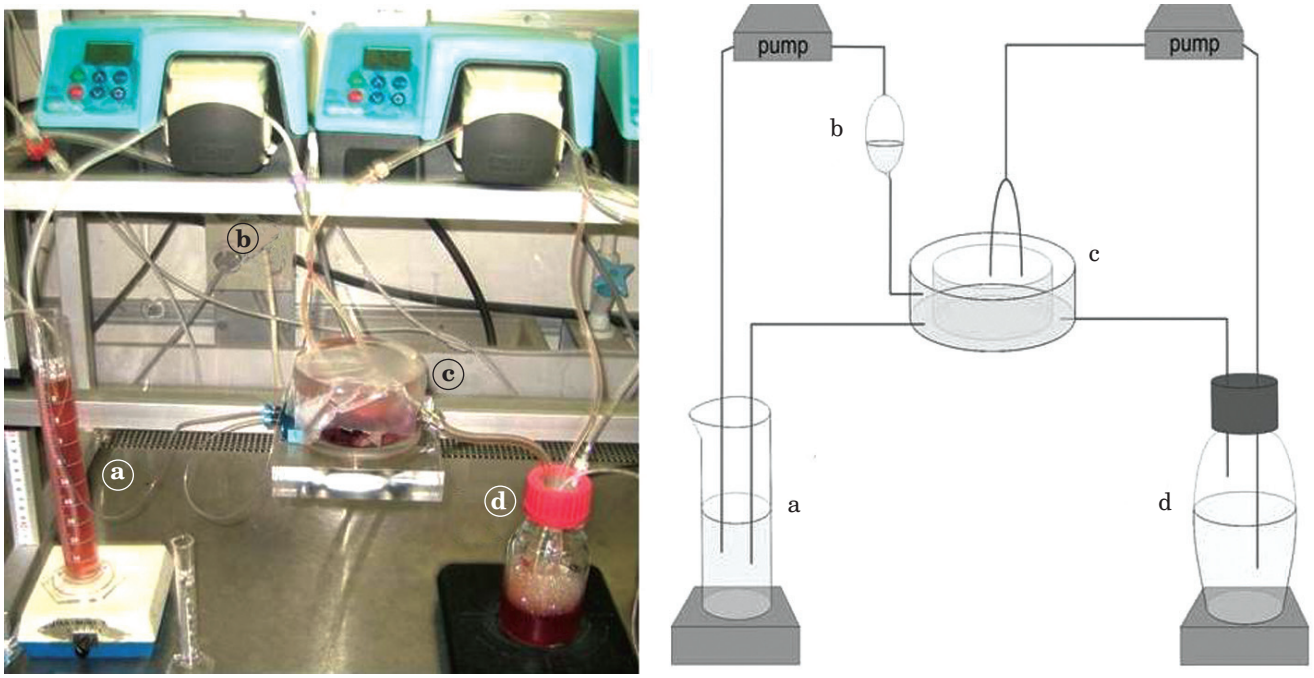
The effects of exposure to parabens, as well as to bisphenol A, were addressed by using markers of male reproductive health, in a study involving male partners requiring treatment in an infertility clinic (8). Results from 167 urine samples suggested that there was no association between the levels of parabens and hormones (FSH, LH, inhibin B, FSH, T, E_2, SHBG, FAI, prolactin). In addition, 190 urine samples were analysed to determine any association between semen quality and parabens levels. Again, the analyses revealed no association. However, a positive association between BuP concentration and sperm DNA damage, measured with a Comet assay on 132 urine samples, was found — for both single and combinations of parabens.

Parabens have also been considered to be factors which may influence the occurrence of breast cancer in women (4, 28). Two studies have been performed to find an association between the use of deodorants and/or antiperspirants and breast cancer, but neither was specifically investigating the effects of parabens. No association between the use of these types of product and breast cancer has been found.

Aims of the proposed study

The transport of exogenous chemicals across the placenta from the mother to the fetus is an important issue, since it could result in detrimental effects on the fetus *in utero* or in later life. It is particularly important to use a system which reproduces accurately the 'real' situation, such as the human placental tissue, avoiding the use of animals or animal-derived tissues, so complications

Figure 2: The placental perfusion set-up



The fetal reservoir (a) is located on the left-hand side and the maternal reservoir (d) on the right-hand side, each on top of a magnetic stirring device. The fetal in-flow tubing contains a bubble trap (b), above which are the two peristaltic pumps. In the centre of the set-up is the perfusion chamber (c). The whole system is located in a heated flow bench that keeps it at a temperature of 37°C.

arising from interspecies differences in placental transport and metabolism can be avoided.

Placentas exhibit a large degree of species specificity, due to anatomical and functional differences. This makes it difficult to interpret animal data with respect to predicting fetal exposure in human pregnancy (29). Simian primates provide the closest models, even though important differences still exist (29, 30). Sheep have also been used as a model of human pregnancy, due to their similar placental development, metabolism and nutrient transport. However, significant structural differences are still evident, which result in the higher impermeability of human placental tissue to lipophilic substances, as compared with that of the sheep (31).

The proposal is to develop a method for estimating fetal exposure, by using a placental perfusion model involving only human-derived materials (32). This article outlines a forthcoming study on four of the most commonly-used parabens and their most common metabolite, *p*-hydroxybenzoic acid, in the human placental perfusion system (33, 34). This will contribute to knowledge on human exposure to parabens, and will help to establish the importance of risk management of exposure to parabens used during pregnancy, particularly in the light of the oxidative stress potential and oestrogenic activity of parabens recently investigated in birth cohorts (Table 1; 8).

We have already identified, experimentally, the appropriate conditions for carrying out the analysis on human placenta samples by using High Performance Liquid Chromatography (HPLC). The method was developed to enable the sensitive and simultaneous detection of MeP, EtP, PrP, BuP and *p*-hydroxybenzoic acid. We aim to validate the method in terms of sensitivity, precision, linearity and selectivity. The relevance of the study also relies on the fact that the procedure can be used to produce comparable data worldwide.

Replacement benefits of using the human placental model:

— the placental perfusion method uses only human tissue;

— species–species extrapolation is unnecessary (a major benefit, as the human placenta is unique among all other species); and

— the human placental perfusion model can potentially replace animals in the study of fetal chemical exposure, a part of reproductive toxicity testing strategies.

The Human Placental Model

The placental perfusion model has been used to test the transplacental transport of various ubiquitous environmental chemicals, including phthalates (35), brominated flame retardants (BFRs; 36), and polychlorinated biphenyls (PCBs; 37). The results from these studies have added important information to the research area of placental transport, as it was not obvious from their chemical structures alone, how the substances were transported across the placenta. The BFRs and PCBs had opposite transport kinetics, according to the extent and nature of their halogenation, in that the BFRs were transported more readily with decreasing bromination of the molecules, and the PCBs were transported more readily with increasing chlorination. This highlights the importance of studying each group of compounds separately, and studying a sufficiently large number of chemicals from each group.

In the study of toxic compounds, the metabolite can be found to be more toxic than the absorbed compound, and this creates the need for studying metabolites. In the placental perfusion study of benzo[*a*]pyrene (BaP; 38), we investigated the placental metabolism of this compound to form a genotoxic product, benzo[*a*]pyrene diolepoxide, during a six-hour perfusion.

An analytical method to detect parabens in placental tissue, by using LC-MS/MS, has been established previously by another research group (39). In the proposed study, we will use a new analysis-method that we have developed, to simultaneously and sensitively quantify the four most widely-used parabens and their main metabolite in the placental perfusate, in order to observe the transport and metabolic activities of the placenta. In addition, there will be an opportunity to carry out more-realistic exposure assessments of parabens, as the analysis-method could be used in the future for new epidemiological studies. This could be of great importance, due to the lack of sufficient data on the effects of parabens and *p*-hydroxybenzoic acid on human health.

No animal ingredients are used in the culture medium or in the production of the samples. Therefore, the *ex vivo* human placental perfusion model is an effective and more-relevant alternative to animal studies, and thus may potentially contribute to the *reduction* and *replacement* of animal testing. National and international collaboration concerning the human placental perfusion model has been established since 2006 (40). Comparisons with human *in vivo* data obtained by human biomonitoring confirm the relevance of this human model as part of *in vitro* toxicology testing strategies (in Denmark and internationally). The research programme, ReProTect, aims to address reliability and relevance in the development of *in vitro* tests for reproductive toxicology (41). Our human placental perfusion studies have been included in this research programme, and it is foreseen that the model will be included as a recognised part of reproductive toxicity testing in the EU.

The human placental perfusion model

The placental perfusion system (Figure 2) uses the human placenta to investigate the transfer of environmental toxicants from the maternal circulation to the fetal circulation, and to determine the metabolism of these chemicals in the placenta. After exposure, the placenta can undergo histological analysis, in order to reveal more about the transport mechanisms and general features of the placenta.

The system can also be used to study different parameters in the placental tissue, including cytotoxicity and alterations in hormone production. Data from our studies on human placental transport can help to characterise the transport of a substance according to its molecular properties (35–38, 42, 43). The method is constantly being improved to create a system as close to the physiological situation as possible.

The *ex vivo* placental perfusion system (Figure 2) was first described and developed by Panigel, and later was modified and refined by Schneider and other research groups (44, 45). Interlaboratory comparisons have validated the system (34, 46). Human placental perfusion requires that the live placental tissue is acquired directly after birth. The human placental perfusion facility is sufficiently close to the maternity ward at Rigshospitalet (Copenhagen University Hospital, Denmark) to obtain viable placentas, with ethical approval and the necessary logistics already in place. The placentas used are donated, with informed written maternal consent, after an uncomplicated pregnancy and vaginal delivery or Caesarean section (35, 40).

In the set-up of the model, the placenta is brought to the laboratory from the maternity ward within 30 minutes. A vascular unit of the placenta is re-perfused by using neonatal feeding tubes (FlocarePurSondes-MP), and, if the fetal vessels are not leaky, the placental unit is cut from the placenta and the maternal adjacent part of the placenta is bluntly cannulated. The placental unit is placed in a 37°C flow chamber, and the fetal and maternal circulations are established (Figure 2) and circulated with perfusion medium (RPMI 1640; Panum Institute, University of Copenhagen, Denmark), supplemented with 1ml L-glutamine (200mM), penicillin and streptomycin (Panum Institute, University of Copenhagen), and physiological levels of Human Serum Albumin (HSA; 20% stock solution; fetal = 40mg/ml; maternal = 30g/ml; CSL Behring GmbH, Marburg, Germany). The fetal perfusion medium is gassed with 95% N_2: 5% CO_2 (v/v), and the maternal medium is gassed with 80% O_2 : 20% CO_2 (KVG, Denmark). After a 30-minute preperfusion, the test substances and the positive control substance are added, and the fetal and maternal circulations are recirculated for 240 minutes. Samples (0.5ml) are taken before the start of the perfusion and after every 30 minutes. After the perfusion, tissue samples (1g) are taken from the cotyledon, and from the non-perfused placenta.

Chemicals, stock solutions and sample preparation

MeP, EtP, PrP and BuP parabens and *p*-hydroxybenzoic acid will be purchased from Sigma-Aldrich Denmark ApS. Antipyrine (Aldrich-Chemie, Steinheim, Germany) will be used as a positive control in the perfusion system: fetal side and maternal side overlap, as this is a small molecule which is transported by passive diffusion. The negative control is leakage from the fetal system.

Stock solutions of parabens and *p*-hydroxybenzoic acid will be prepared by dissolving an appropriate amount of the test compounds in ethanol, without any additional purification of the chemicals. Standard solutions (1mM) will be prepared from the stock solutions, by further dilution with ethanol.

The perfusion samples will be centrifuged for 5 minutes at 4,000*g*, then the supernatant will be transferred to a vial and stored at –20°C until required for antipyrine and paraben determination by HPLC and UV output detection. Tissue samples will be extracted in 1ml of ethanol for at least 24 hours, and a 200µl aliquot will subsequently be analysed by HPLC.

Sample analysis

The HPLC system used in the laboratory is the Hitachi LaChrom (Merck, Japan), incorporating an UV-Vis detector (Hitachi, L7400, Japan). The stationary phase is a C18 column (Nucleosil, ODS; 20Å ~4.6mm ID, 5µm particles; Düren, Germany), with a SecurityGuard precolumn (Phenomenex, ODS; 4Å ~3mm ID; Allerød, Denmark). The internal standard, phenol (Bie & Berntsen AS, Rødovre, Denmark), was included in each analysis.

Antipyrine and parabens will be separated by a C18 column at an oven temperature of 30°C, with an isocratic elution program at a flow rate of 0.9ml/min. The mobile phase will consist of degassed acetonitrile and water (70:30 v/v solution). Injection volumes will be 20–25µl, and the detection absorbance will be 250nm. Peaks will be identified by comparison of R_t and UV absorption spectra with the standards of parabens and *p*-hydroxybenzoic acid (47). Quantitative determinations will be performed by using standard curves.

Conclusions

Although there is much concern about the safety of products containing parabens — and especially cosmetics and personal care products — at present, there are not sufficient data of direct relevance to humans to permit reliable risk assessments. One important question is the extent to which parabens are metabolised in the placenta, and the extent to which they and their metabolites cross the placenta to enter the fetal circulation. It is hoped that the proposed study, involving the human placental perfusion model and supported by the 2012 Lush Young Researcher Prize award to Line Mathiesen, will provide a new and useful answer to this question.

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