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[1] ***Abbreviations***

BPS: Bisphenol S; BPA: Bisphenol A; BPE: Bisphenol E; 3-3BPA: 2,2-Bis(4-hydroxy-3-methylphenyl)propane (3-3BPA); BPB: Bisphenol B; BPC: Bis(4-hydroxyphenyl)-2,2-dichloroethylene; BPBP: Bisphenol BP; BPF: Bisphenol F; BPFL: Bisphenol FL; BPZ: Bisphenol Z; BP4-4: 4,4′-Dihydroxybiphenyl; BPAP: Bisphenol AP; BPAF: Bisphenol AF; BPP: Bisphenol P; BPM: Bisphenol M; LOQ: limit of quantification.

BSA: Bovine Serum Albumin; ESI- : negative electrospray; IS: Internal Standard; MRM: Multiple Reaction Monitoring; OAT: Organic Anion Transporter; QC: Quality Control; RCR: Relative Concentration Residuals; UHPLC-MS/MS: Ultra-High-Performance Liquid Chromatography method coupled to tandem mass spectrometry[1]

Highlights

- The efficiency of placental transport differs greatly between bisphenols.
- BP4-4, BPAP, BPE, BPF, 3-3BPA, BPB, BPA cross placenta by simple diffusion.
- Materno-fetal transfer of BPFL and BPS is very limited.

Abstract

Regulatory measures and public concerns regarding bisphenol A (BPA) have led to its replacement by a variety of alternatives in consumer products. Due to their structural similarity to BPA, these alternatives are under surveillance, however, for potential endocrine disruption. Understanding the materno-fetal transfer of these BPA-related alternatives across the placenta is therefore crucial to assess prenatal exposure risks.

The objective of the study was to assess and compare the placental transfer of a set of 15 selected bisphenols (BPs) (BP 4-4, BPA, BPAF, BPAP, 3-3 BPA, BPB, BPBP, BPC, BPE, BPF, BPFL, BPM, BPP, BPS and BPZ) using the *ex vivo* human placental perfusion model.

The UPLC–MS/MS method for simultaneous quantification of these BPs in perfusion media, within a concentration range of 0.003 to 5 µM, was able to measure placenta transfer rates as low as 0.6 % to 4 %. Despite their structural similarities, these BPs differed greatly in placental transport efficiency. The placental transfer rates of BP4-4, BPAP, BPE, BPF, 3-3BPA, BPB, BPA were similar to that of antipyrine, indicating that their main transport mechanism was passive diffusion. By contrast, the placental transfer rates of BPFL and BPS were very limited, and intermediate for BPBP, BPZ, BPC, BPM, BPP and BPAF, suggesting weak diffusional permeability and/or that their passage might involve efflux transport. These placental transfer data will be particularly useful for predicting the fetal exposure of this important class of emerging contaminants.

Keywords: bisphenols, human placental transfer, mixture, endocrine disruptor, Liquid chromatography, Mass spectrometry

Introduction

Serious concerns regarding widespread human exposure to Bisphenol A (BPA) and its adverse health effects have led to the ban or restriction of BPA production and use in many countries, prompting the development of structural analogues to replace BPA in a variety of industrial products (Chen et al., 2016; Liao and Kannan, 2013; Yang et al., 2014). The list of such BP alternatives is still growing and over 20 BPs, including bisphenols AF, AP, B, F, P, S and Z, are used as monomers in the manufacture of epoxy resin and polycarbonate for many consumer products, such as foodstuffs, personal care products, and paper products (Chen et al., 2016; González et al., 2020; Liao and Kannan, 2014). Given the environmental ubiquity of these BPs, humans are constantly being exposed via dietary intake and daily dermal contact with such products. The increased frequency of detection of BPA alternatives in urine indicates the reality of BPA substitution (Frederiksen et al., 2020; Gyllenhammar et al., 2017; Husøy et al., 2019; Lehmler et al., 2018; Lucarini et al., 2020; Philips et al., 2018; Ye et al., 2015). In particular, a human biomonitoring study revealed the presence of 7 BPs (detection rate: BPA 93%, BPS 89%, BPF 12%, BPP 39%, BPZ 18%, BPAF 66% and BPAP 44%) in 283 urine samples collected from children in southern China (Chen et al., 2018).

However, little is known about the potential adverse effects of these emerging BPA substitutes and most of the toxicological information is limited to their endocrine disruption potential. Like BPA, these structurally similar phenolic compounds seem to affect hormonal systems (Chen et al., 2016; Kojima et al., 2019; Moreman et al., 2017; Pelch et al., 2019; Rochester and Bolden, 2015; Rosenmai et al., 2014; Siracusa et al., 2018; Usman and Ahmad, 2016; Zhang et al., 2018). The developmental effects of some BPs analogues in mammals, mainly rodents, have been documented in a recent literature review (Pelch et al., 2019). Most studies have only investigated a single BPA analogue, such as BPS, BPAF, BPF or BPE, thereby precluding direct comparisons between them. In addition, the endpoints evaluated in these studies were diverse and included altered hormonal signaling and reproduction (Shi et al. 2018), disruption of mammary gland development (Tucker et al., 2018), behavioral changes (Catanese and Vandenberg, 2016) and

77 metabolic disorder (Mustieles et al., 2020). For example, a study of prenatal *in vivo* exposure to
78 BPS and BPAF showed that such exposure affects the mammary gland of the developing fetus
79 (Tucker et al., 2018). Altogether, these data highlight the vulnerability of the developmental period
80 to exposure to these emerging BPs.

81 Data on human biomonitoring of BP analogues in maternal-cord blood samples remain limited, with
82 most studies focused on only 2 or 3 analogues (Ihde et al., 2018; Liu et al., 2017; Pan et al., 2020;
83 Zhang et al., 2020). Several alternative BPs, including BPS, BPAF, BPE, BPF and BPAP have
84 frequently been detected in the human maternal-fetal-placental unit (Pan et al., 2020), indicating
85 that such contaminants can across the placental barrier. Given their structural diversity, the harmful
86 consequences of BPA substitution for fetal health may be exacerbated if the physicochemical
87 properties of these analogues promote their placental transfer, and thus increase fetal exposure.
88 Understanding the materno-fetal transfer of these BPA alternatives across the placenta is therefore
89 crucial for assessing prenatal exposure risks.

90 To that end, the *ex vivo* perfused human placental cotyledon model, which allows reproduction of
91 the conditions of the third trimester of pregnancy, is the method of choice for studying human
92 placental transfer (Hutson et al., 2011). This model had already been used to quantify the
93 transplacental transfer of BPA (Corbel et al., 2014) and BPS (Grandin et al., 2019) and was
94 therefore used here to investigate the materno-fetal placental transfer of fifteen BPs. We focused
95 on bisphenol 4-4, bisphenol A, bisphenol AF, bisphenol AP, 3-3 bisphenol A, bisphenol B,
96 bisphenol BP, bisphenol C, bisphenol E, bisphenol F, bisphenol FL, bisphenol M, bisphenol P,
97 bisphenol S, and bisphenol Z (Figure 1), because of their occurrence in foodstuffs (Caballero-
98 Casero et al., 2016; Liao and Kannan, 2013), their volume of production ("Search for Chemicals -
99 ECHA,"), their presence in human urine (Chen et al., 2018) and their structural diversity.

100 We chose a cocktail approach because the placenta perfusion method remains a time-consuming
101 experimental method that required some logistic and technical challenges. We first developed and
102 validated an ultra-high-performance liquid chromatography method coupled to tandem mass
103 spectrometry to assay all fifteen BPs simultaneously. To check that the combination of 15 BPs did

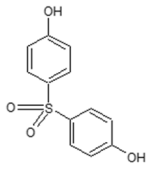
not interfere with the placental transfer mechanism, we compared the maternal-fetal placental indices of BPA and BPS evaluated with this cocktail approach to those previously determined for these compounds perfused individually using the same model (Corbel et al., 2014; Grandin et al., 2019). This approach, by allowing comparison of the values obtained for a key parameter, placental clearance, should provide new insights for understanding fetal BPs exposure and assessing the hazards of BPA substitution in vulnerable populations, such as the developing fetus.

1. Material and methods

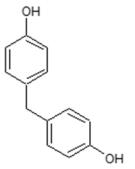
The study was designed to evaluate the simultaneous placental transfer of fifteen BPs in the maternal-fetal direction.

1.1 Chemicals

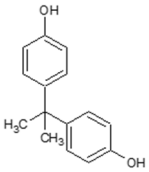
Bisphenol S (BPS) (purity ≥ 98%), Bisphenol A (BPA) (purity ≥ 99%), Bisphenol E (BPE) (purity ≥ 98%), 2,2-Bis(4-hydroxy-3-methylphenyl)propane (3-3BPA) (purity ≥ 97%), Bisphenol B (BPB) (purity ≥ 98%), Bis(4-hydroxyphenyl)-2,2-dichloroethylene (BPC) (purity ≥ 98%), Bisphenol BP (BPBP)(purity ≥ 98%), Bisphenol F (BPF) (purity ≥ 98%), Bisphenol FL (BPFL) (purity ≥ 97%), Bisphenol Z (BPZ) (purity ≥ 98%), 4,4′-Dihydroxybiphenyl (BP4-4) (purity ≥ 97%), Bisphenol AP (BPAP) (purity ≥ 99%), Bisphenol AF (BPAF) (purity ≥ 97%), Bisphenol P (BPP) (purity ≥ 98%), Bisphenol M (BPM) (purity ≥ 99%) (Figure 1) and antipyrine (purity ≥98%) were purchased from Sigma-Aldrich (Saint-Louis, Missouri, USA).



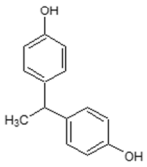
4,4'-sulfonyldiphenol ***(BPS)***
M= 250 g/mol
CAS : 80-09-1



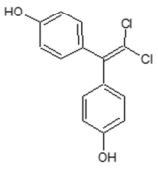
4,4'-methylenediphenol
(BPF)
M=200g/mol
CAS : 620-92-8



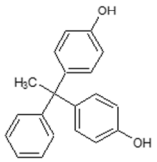
4,4'-(propane-2,2-
diyl)diphenol ***(BPA)***
M=228 g/mol
CAS : 80-05-7



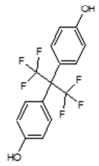
4,4'-(ethane-1,1-
diyl)diphenol ***(BPE)***
M=214 g/mol
CAS : 2081-08-05



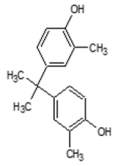
4,4'-(2,2-dichloroethene-1,1-
diyl)diphenol
(BPC)
M=280 g/mol
CAS : 14868-03-2



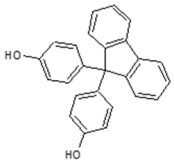
4,4'-(1-phenylethane-1,1-
diyl)diphenol
(BPAP)
M=290 g/mol
CAS : 1571-75-1



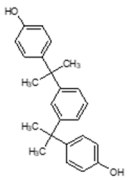
4,4'-(perfluoropropane-2,2-diyl)
diphenol ***(BPAF)***
M=336 g/mol
CAS : 1478-61-1



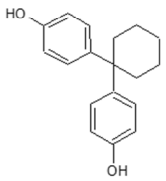
4,4'-isopropylidenedi-0-
cresol
(3-3 BPA)
M=256 g/mol
CAS : 79-97-01212



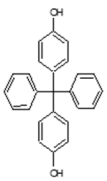
4,4'-(9H-fluorene-9,9-diyl)
Diphenol ***(BPFL)***
M= 250 g/mol
CAS : 3236-71-3



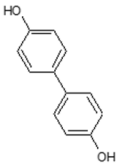
4,4'-(1,3-phenylenebis
(propane-2,2-diyl))diphenol ***(BPM)***
M=246 g/mol
CAS : 13595-25-0



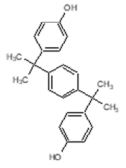
4,4'-(cyclohexane-1,1-diyl)
diphenol ***(BPZ)***
M=268 g/mol
CAS : 843-55-0



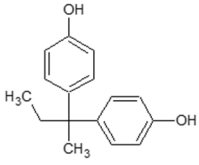
4,4'-(diphenylmethylene)
diphenol ***(BPBP)***
M=352 g/mol
CAS : 1844-01-5



[1,1'-biphenyl]-4,4'-diol
(BP4-4)
M=186 g/mol
CAS : 92-88-6



4,4'-(1,4-
phenylenebis(propane-2,2-
diyl))
diphenol ***(BPP)***
M=346 g/mol
CAS : 2167-51-3



4,4'-(butane-2,2-diyl)diphenol
(BPB)
M=242 g/mol
CAS : 77-40-7

123

124 Figure 1: Molecular structures of the fifteen BPs

125 Six isotopes-labeled standards, namely, BPAF-d4 (purity ≥ 98%), BPAP-d5 (purity ≥ 97.5%), BPP-
126 d16 (purity ≥ 98%), BPS-d8 (purity ≥ 97%), and BPA$^{13}C_{12}$ (purity ≥ 98%), used as internal
127 standards, were purchased from Toronto Research Chemicals (Toronto Canada). Methanol and
128 Acetonitrile were LC/MS quality and purchased from Fisher Scientific (Illkirch, France) and acetone
129 was LC quality purchased from Sigma-Aldrich (Saint-Louis, Missouri, USA).

130 All BPs, except BPBP, were dissolved in ethanol at a concentration of 150mM. BPBP was
131 dissolved at 30mM, due to its weak solubility. A solution containing all fifteen BPs was prepared at
132 the same concentration of 7.5mM for each BP, except BPBP (concentration of 1.5mM). The
133 solutions were stored at -20 °C until use. Bovine serum albumin (BSA, Fraction V, purity 100%,
134 Aviva Systems Biology, San Diego, USA) and Earle's Balanced Salt solution were purchased from
135 United States Biological (Salem, MA 01970, United-States). The materials for placental perfusion,
136 including the materials used to prepare solutions, sampling, processing and analysis, were in glass
137 or BPA- and BPS-free plastic containers and the absence of leaching of BPs from the tubing was
138 verified.

139 The absence of binding to the perfusion equipment was checked by comparing the BPs
140 concentrations in the maternal reservoir and in the maternal perfusate circulating in the tubing
141 without the cotyledon (mean ratios between 93 and 103% for the BPs).

142 1.2 Placental perfusion

143 Placentas (519 ± 113g) were collected from HIV-seronegative women with normal pregnancies
144 following vaginal (n = 1) or caesarean (n = 4) delivery in the CHU Paule de Viguier, Toulouse,
145 France. The study received institutional approval (DC-2016-2694) and each patient gave written
146 informed consent to participate in the study.

147 Collected placentas were perfused in an open double circuit as previously described (Corbel et al.,
148 2014; Grandin et al., 2019). Perfusion experiments were started within 30 min after delivery.
149 Briefly, after visual confirmation of the vascular integrity of both the maternal and fetal sides, a
150 distal branch of a fetal artery and the associated vein supplying a peripheral cotyledon, were

151 cannulated (Microtube Tygon S54HL, Saint Gobain, Courbevoie, France). The balance between
152 arterial and venous fetal flows was evaluated and placentas with evidence of vascular leakage
153 were discarded (Karttunen et al., 2015). On the maternal side, the perfused area progressively
154 whitened, which allowed visualization of the chosen cotyledon. The cotyledon was placed in the
155 perfusion chamber and maintained at 37°C, with the maternal side upward. Perfusion on the
156 maternal side was subsequently initiated by inserting two catheters into the intervillous space. The
157 fetal and maternal flows were 6 and 12 ml/min, respectively, and the perfusion length was 90 min.
158 The pH was continuously adjusted throughout the perfusion to 7.41 ± 0.022 and 7.23 ± 0.025 for
159 the maternal and fetal perfusion media, respectively. The balance between arterial and venous
160 fetal flows and pressures (between 40 and 60 mm Hg) were monitored throughout the experiment
161 to control the integrity and functionality of the perfusion. Earle medium supplemented with 25 g/L
162 bovine serum albumin (BSA) at 37 °C was used to reflect the physiological plasma protein
163 concentrations at late pregnancy (Larsson et al., 2008). Indeed, some BPs (BPA, BPS, BPF, BPE,
164 BPB, BPM and BPAF) have been shown to bind to plasma albumin (Grumetto, 2019; Luo et al.,
165 2016; Wang et al., 2014; Yang et al., 2017). BSA was used because of its 76.5 % sequence
166 homology with human serum albumin. Antipyrine (20 µg/mL), a reference substance for control of
167 passive diffusion and barrier integrity (Karttunen et al., 2015), and the BPs mixture, were added to
168 the maternal reservoir to study materno-fetal transfer. Because the placental transfer of BPS had
169 been previously reported to be low (Grandin et al., 2019), all BPs (except the weakly soluble
170 BPBP) were used at a final nominal concentration of 5 µM (1 µM for BPBP) to ensure their
171 effective detection in the fetal compartment.

172 Control samples (1 mL) were collected from the fetal and maternal inflow reservoirs before the
173 addition of antipyrine and BPs and from the maternal reservoir after simultaneously adding BPs
174 and antipyrine solutions (time 0) and at 30, 60 and 90 min. During the perfusion, fetal exudates
175 were collected every 5 min and their volumes measured. Maternal exudates were collected
176 immediately after adding the test molecules and every 30 min up to 90 min. At the end of the
177 perfusion, the isolated cotyledon was rinsed with phosphate-buffered saline (pH 7.4) for 15 min.

178 This washing solution was collected. All samples were immediately chilled in ice and centrifuged
179 for 10 min at 3000g and 4 °C to discard placental cells and the supernatant was collected and
180 stored at -20 °C until assayed.

181 1.3 Antipyrine assay

182 Antipyrine concentrations in the perfusion medium were determined by UHPLC coupled with UV
183 detection. Briefly, antipyrine was extracted from the perfusion medium (100 µL of sample) by
184 adding 200µL of 5 % trichloroacetic acid. After mixing and centrifuging (20000 g, 10 min and 4°C),
185 samples were eluted on a C18 column at 0.3 mL/min and 40°C using H_2O / acetonitrile gradient
186 elution with UV detection set at 250 nm. The calibration curve ranged from 0.5 to 50 µg/mL. Data
187 were fitted using a linear model with 1/X (X = concentration) as weighting factor. Blank samples
188 were used to check the absence of contamination during the assays. Accuracy ranged from 87% to
189 103% and the intra- and inter-day coefficients of variation for three concentration levels (0.8, 8 and
190 30 µg/mL) were below 11%. The limit of quantification (LOQ) was validated at 0.5 µg/mL.

191 1.4 Simultaneous quantification of 15 bisphenols in placenta perfusion media

192 1.4.1 Analytical conditions

193 The materno-fetal transfer rates of BPA (Corbel et al., 2014) and BPS (Grandin et al., 2019)
194 previously evaluated on the same perfused placenta model were on average 29 and 3.18 %,
195 respectively. Our objective was to develop a simultaneous assay, without dilution, of the fifteen
196 BPs both in the maternal reservoir and in the fetal outflow perfusate, i.e. with a wide concentration
197 range between 0.05 and 5 µM so as to be able to evaluate materno-fetal transfer rates of BPs
198 ranging between 1% and 40-50%.

199 Samples were assayed by ultra-high performance liquid chromatography coupled to tandem mass
200 spectrometry (Acquity-2D UPLC® Xevo® TQ, Waters, Milford, MA, USA) using the methods
201 previously described by Grandin et al., 2017 and Lacroix et al., 2011. Briefly, BPs were extracted
202 from the perfusion medium (100µL of sample) by adding 200µL of acetone containing internal

standards (100 ng/mL). Samples were mixed for 1 min at 10°C and 1400 rpm and centrifuged at 20000 g and 4°C for 10 min. Then, 200 µL of supernatant were evaporated to near dryness under nitrogen at 50°C and reconstituted with 200µL of methanol/H_2O: 50/50 (v/v). The BPs were separated on a Raptor Biphenyl column (100 x 2.1 mm; 2.7µm, Restek) at 0.3 mL/min and 40°C using H_2O/ methanol gradient elution.

Analytes were detected in negative electrospray (ESI^-) using multiple reaction monitoring (MRM) mode. The MRM transitions of BPs and IS with their respective cone voltages and collision energies are given in Supplementary Material Table S1.

1.4.2 Validation procedure

The performance of the method was evaluated according to the European Medicine Agency Guidelines (European Medicine Agency, 2011) for bioanalytical method validation in terms of linearity, intra-day and inter-day repeatability, and sensitivity. Selectivity was tested by comparing five blank perfused media chromatograms with chromatograms at the limits of quantification (LOQ). The LOQs were defined as the lowest concentrations of the calibration curve that could be quantified with less than 20% coefficient of variation (CV%), precision and within an accuracy range of 80–120%.

For each BP calibration curve, both simple ($Y = aX + b$) and quadratic ($Y = aX^2 + bX + c$) models were tested after applying appropriate weighting: 1, $1/X$ and $1/X^2$ (X = nominal concentration). Three approaches were adopted to assess the linearity of the calibration curve: (1) calculation of the relative concentration residuals (RCR%) between the nominal concentration and the concentration obtained with the model, which should be lower than ± 15% (except at the LOQ, ± 20%), (2) visual inspection of the residual distribution which should be randomized around the mean and (3) application of a lack-of-fit test to check the goodness-of-fit of the model. Within-day and between-day precisions and accuracies for each BP were calculated on three different days and with six replicates of QC samples at four concentration levels (3, 30, 300 and 1500 ng/mL).

1.5 Placental transfer parameters

Perfusions in the materno-fetal direction were validated if the transfer rate of antipyrine was above the generally accepted threshold of 20% (Challier et al., 1983; Gavard et al., 2009; Schneider et al., 1972) and remained stable throughout the perfusion. Only the concentrations at steady state for antipyrine and all BPs were used to calculate the transfer rate and the clearance index.

As previously described (Corbel et al., 2014; Grandin et al., 2019), the transfer rate was calculated for each steady state time point as the ratio between concentrations in the fetal compartment to concentrations in the maternal compartment. To take the inter placenta variability into account, the clearance index *i.e.* the ratio of the transfer rate of each bisphenol to that of antipyrine, was determined.

The mass balance for antipyrine and the 15 BPs was calculated as the ratio of the sum of the quantities of substrate in all the maternal and fetal exudate media and PBS washings to the measured amount of substrate in the maternal reservoir, as previously described (Corbel et al., 2014).

1.6 Statistical analysis

First, the materno-fetal placenta transfer rate of each bisphenol was compared with that of antipyrine using a Dunnett's multiple comparisons test, with a confidence level of 0.95. Two groups of bisphenols had been identified in a first test: one group containing bisphenols with a transfer rate non significantly different to that of antipyrine and a second group containing bisphenols with a significantly lower transfer rate than that of antipyrine. For each group of bisphenols, the placental clearance indices were compared using an ANOVA followed by a post-hoc Tukey test with a confidence level of 0.95. The statistical analyses were done using the R® software (R development core team, 2009).

2. Results

2.1 Performance of the method for the simultaneous quantification of 15 bisphenols

The selectivity of the method was evaluated by comparing the areas of the BPs on five blank samples to the areas at the LOQs. The areas in blank samples were below 20% of that of the LOQs for all BPs except BPAF (29 %) and BPS (43 %). Indeed, BPS was systematically detected in blank media but the areas were always at least two-fold lower than that of their LOQ. However, the method remains suitable for the assessment of BPs placenta transfer since BPs contamination was monitored by injecting blank medium during the sample assays. The LOQs were validated at 1 ng/mL for BPAF and BPAP, 5 ng/mL for BP4-4, BPF, BPE, BPA, BPB, BPZ, BPM, BPFL, BPBP, 10 ng/mL for BPS and 50 ng/mL for 3-3BPA, BPC and BPP, with intra-day CVs below 21 % and accuracies within 83-119%. Intra- and inter-day precisions were below 22% with accuracy ranging from 85% to 117% for all BPs (Table 1). The calibration curve ranges were established from the BP LOQs up to 2000 ng/mL to ensure placental transfer rate measurements between 1 and 50 % without requiring sample dilutions. The method was therefore suitable for the simultaneous quantification of fifteen BPs in both the maternal reservoir and the fetal outflow perfusate.

266

Compound		BP4-4	BPS	BPF	BPE	BPA	BPAF	BPB	3-3BPA	BPZ	BPC	BPAP	BPP	BPM	BPFL	BPBP
Internal Standard		BPA13C12	BPSd8	BPAF-d4	BPAF-d4	BPA13C12	BPAF-d4	BPA13C12	BPAP-d5	BPAP-d5	BPAF-d4	BPAP-d5	BPP-d16	BPAP-d5	BPAP-d5	BPAP-d5
Model		Linear $1/X^2$	Linear $1/X^2$	Linear $1/X^2$	Linear 1/X	Linear 1/X	Linear $1/X^2$	Linear $1/X^2$	Linear 1/X	Linear 1/X	Linear 1/X	Linear 1/X	Linear $1/X^2$	Quadratic $1/X^2$	Quadratic $1/X^2$	Quadratic $1/X^2$
LOQ (ng/mL)		5	10	5	5	5	1	5	50	5	50	1	50	5	5	5
CV intra-day %	QC = 3 ng/mL	-	-	-	-	-	7	-	-	-	-	12	-	-	-	-
	QC = 30 ng/mL	14	4	16	13	18	3	10	-	9	-	5	-	15	9	15
	QC = 300 ng/mL	12	3	13	8	11	4	7	9	4	7	2	13	9	8	5
	QC = 1500 ng/mL	14	2	10	11	9	11	11	8	4	4	3	15	11	14	13
CV inter-day %	QC = 3 ng/mL	-	-	-	-	-	6	-	-	-	-	14	-	-	-	-
	QC = 30 ng/mL	14	4	15	13	22	3	16	-	11	-	8	-	18	21	15
	QC = 300 ng/mL	15	6	14	7	15	4	12	14	4	17	4	16	15	10	9
	QC = 1500 ng/mL	14	4	14	16	14	6	12	16	4	18	3	12	18	15	14
Accuracy %	QC = 3 ng/mL	-	-	-	-	-	105	-	-	-	-	109	-	-	-	-
	QC = 30 ng/mL	97	100	98	95	89	101	109	-	99	-	100	-	117	106	112
	QC = 300 ng/mL	105	102	106	106	96	108	112	103	104	90	101	102	108	113	113
	QC = 1500 ng/mL	114	94	109	97	92	109	97	103	99	85	99	102	90	102	92
LOQ	CV intra-day %	12%	7%	15%	7%	15%	11%	11%	9%	13%	17%	21%	15%	11%	1%	5%
	Accuracy %	114%	105%	113%	99%	106%	109%	100%	119%	116%	94%	111%	108%	83%	94%	103%

267 Table 1: Validation results for the simultaneous quantification of 15 bisphenols in placenta medium.

2.2 Placental transfer of the mixture of 15 bisphenols

The overall mean antipyrine transfer rate, for all the validated perfusions (n=5), was 31.7 ± 12.6% and conforms closely to values reported previously (Corbel et al., 2014; Grandin et al., 2019; Gavard et al., 2009). The wet weight of the perfused cotyledons was 27 ± 4 g (n=3). The average flow rates were 12.5±1.9 mL/min in the maternal circulation and 5.3±0.7 mL/min in the fetal circulation and remained stable throughout the perfusion. No BPs and no antipyrine were detected at levels above the LOQs in control perfusion medium samples collected from the maternal and fetal exudates before adding the compounds. The mean recovery for all BPs was not significantly different from that of antipyrine (89±6.2%, range 86%-91%), except for BPE (77 ± 6 %, range:70-84%, p=0.03, Dunnett test). This incomplete recovery of BPE could be explained by BPE placental metabolism. But the absence of signal corresponding to the MRM transitions of the 15 bisphenol mono-glucuronides confirms the very limited *in vitro* placental metabolism in this open non-recirculating perfusion model.

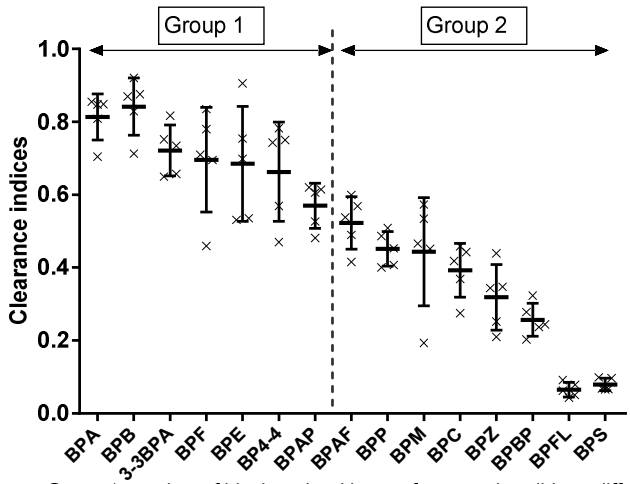
Table 2 summarizes the materno-fetal placental transfer rate and clearance indices of the perfused mixture of fifteen BPs (at the concentration of 5 µM, except for BPBP, 1 µM) and antipyrine (at the concentration of 20 µM).

	n=5	Antipyrine	BPA	BPS	BPF	BPAF	BPB	BPE	BPC	BP4-4	3-3BPA	BPAP	BPBP	BPP	BPM	BPZ	BPFL
Transfer rate[a] (%)	Mean	31.68	25.70	2.45	22.52	16.38	26.45	21.52	12.25	21.13	22.86	18.10	7.99	14.68	14.48	9.87	1.98
	SD	12.64	9.85	0.77	11.10	5.95	9.11	8.86	4.55	9.27	8.75	7.15	2.66	6.68	7.34	3.94	0.70
Placental clearance[b] (mL/min)	Mean	1.671	1.345	0.129	1.164	0.857	1.387	1.115	0.640	1.105	1.196	0.946	0.420	0.770	0.763	0.518	0.105
	SD	0.614	0.467	0.039	0.518	0.284	0.436	0.401	0.222	0.455	0.414	0.341	0.135	0.326	0.367	0.195	0.039
Clearance index[c]	Mean	-	0.813	0.079	0.696	0.522	0.842	0.685	0.392	0.663	0.722	0.570	0.257	0.451	0.444	0.318	0.065
	SD	-	0.063	0.017	0.144	0.072	0.079	0.158	0.074	0.136	0.070	0.062	0.046	0.047	0.148	0.090	0.020

Table 2: Materno-fetal parameters (clearance, transfer rate and clearance index) of 15 bisphenols perfused as a cocktail. [a]The ratio of the concentration in the receiving compartment to the concentration in the entrance compartment expressed as a percentage. [b]The concentration in the receiving compartment multiplied by its flow rate to the concentration in the entrance compartment. [c]The ratio of the transfer rate of each bisphenol to that of antipyrine

290 Despite their similar phenolic structures, these BPs showed a considerable range of placental
291 transfer rates. The transfer rates of BP4-4 (21.13 ± 9.27 %), BPAP (18.10 ± 7.15 %), BPE (21.52 ±
292 8.86 %), BPF (22.52 ± 11.10 %), 3-3BPA (22.86 ± 8.75 %), BPB (26.45 ± 9.11 %) and BPA (25.70
293 ± 9.85 %) were not significantly different to that of freely diffusible antipyrine (31.68 ± 12.64%). By
294 contrast, the placental transfer rates of the others were significantly lower than that of antipyrine
295 ($p<0.05$, Dunnett's multiple comparisons test) and ranged from 7-16% for BPBP (7.99 ± 2.66%),
296 BPZ (9.87 ± 3.94%), BPC (12.25 ± 4.55%), BPM (14.48 ± 7.34%), BPP (14.68 ± 6.68%) and BPAF
297 (16.38 ± 5.95%). In addition, the BPFL and BPS placental transfer rates were 10 and 13-fold lower
298 than that of BPA, *i.e.*, 1.98 ± 0.70 % and 2.45 ± 0.77 %, respectively.

299 Figure 2 depicts the individual values and mean ± SD of the BP clearance indices. The clearance
300 indices for BPs ranged from 0.065 to 0.842. In group 1, the clearance indices were very close and
301 only BPA and BPB had a significantly higher clearance index than BPAP (Tukey test). In group 2,
302 the clearance indices gradually decreased from 0.522 (BPAF) to 0.257 (BPBP), whereas the
303 clearance indices of BPFL (0.065) and BPS (0.079) were significantly lower than those of all BPs in
304 Group 2. The BPBP clearance index was significantly lower than those of BPM, BPP and BPAF
305 and the BPAF clearance index was significantly higher than that of BPZ.



Group 1 consists of bisphenols with transfer rates that did not differ significantly from that of antipyrine.
Group 2 consists of bisphenols with transfer rates that differed significantly from that of antipyrine.

306

307 Figure 2: Clearance indices of the 15 BPs (individual values (x) and mean ± SD), corresponding to
308 the ratio of BP-studied placental transfer rate divided by the antipyrine transfer rate.

3. Discussion

Prenatal exposure to BPs may result in adverse effects on sensitive developmental processes. The scarcity of data concerning exposure of the human fetus to BPs analogues highlights the urgent need for a better understanding of the maternal-fetal-placental exchanges of these emerging structurally-related BPs.

In the present study, the *ex vivo* perfusion model of the human term placenta was used to examine the placental passage of 15 structurally related BPs. A method for the simultaneous quantification of 15 BPs analogues in the perfusion media was developed in UHPLC-MS/MS with performance fulfilling the acceptability criteria recommended in the EMA guidelines, in terms of sensitivity, precision and accuracy (European Medicines Agency, 2011). This method is suitable for quantifying low materno-fetal placental transfer rates within 0.6-4% depending on the BPs.

Antipyrine, a free passive diffusion reference which is not bound to plasma proteins, was used as a reference substance to assess the transfer rates of lipid-soluble xenobiotics (Ala-Kokko et al., 2000). The antipyrine clearances observed in this study (1.06-2.34 mL/min) were close to values reported previously, 2.24±0.68 mL/min and 1.83±0.619 mL/min, respectively (Corbel et al., 2014; Grandin et al., 2019). The clearance of each BP was expressed as a fraction of the antipyrine clearance (clearance index) to overcome inter-placental variability (Challier, 1985), and allow comparisons between different BPs and different experiments.

The BPA and BPS clearance indices (0.813±0.063 and 0.079±0.017) evaluated here with a combination of fifteen BPs, did not differ from those determined previously with the same compounds perfused separately (0.813±0.13 and 0.0852±0.0515, respectively, (Corbel et al., 2014; Grandin et al., 2019), suggesting that the combination of 15 BPs did not interfere with the placental transfer mechanism and thus validated our cocktail approach.

Despite the structural similarities of these 15 BPs, the clearance indices for the current data set ranged from 0.065 for BPFL to 0.842 for BPB, indicating considerable differences in the efficiency of BP transport between BPs. The placental transfer rates of BP4-4, BPAP, BPE, BPF, 3-3BPA,

BPB, BPA were similar to that of antipyrine, suggesting that exchange of these bisphenols across the placenta mainly involves passive diffusion and is solely limited by the placental blood flow. By contrast, the placental transfer rates for BPFL and BPS were very limited, and intermediate for BPBP, BPZ, BPC, BPM, BPP and BPAF suggesting that the placental transfer of these BPs does not rely solely on weak diffusional permeability but might involve differentially active transport. However, considering the high doses of BPs used, a saturation of the placental transfer cannot be ruled out.

In agreement with these findings, previous comparative toxicokinetic results in a pregnant sheep model showed that the materno-fetal transfer of BPS was ten-fold lower than that of BPA (Gauderat et al., 2017; Grandin et al., 2018) and that the feto-maternal total concentrations ratio of BPS was lower than those of BPF and BPA after a single subcutaneous administration of BPS or a mixture of BPA, BPS and BPF (Gingrich et al., 2019). Moreover, in a recent prospective birth cohort (Zhang et al., 2020), the ratio between umbilical cord and maternal total plasma concentrations of BPS was lower (1.11 in 11 pairs) than the ratios for BPA (1.94, n=73) and BPAF (3.26, n=22), which is consistent with a lower placental passage of this BP analogue. However, the human isolated cotyledon model, unlike the *in vivo* model, does not incorporate those nonplacental toxicokinetic factors that determine fetal exposure, such as maternal and fetal metabolism. Thus, it was shown in the pregnant sheep model that despite the low materno-fetal placental transfer of BPS, the accumulation of BPS in the fetal compartment after repeated maternal exposure led to chronic fetal exposure to BPS in a range of concentrations similar to those obtained for BPA (Grandin et al., 2018). Another limitation imposed by this model is that the present results refer to placenta at term and cannot necessarily be extrapolated to earlier stages of gestation. However, due to the placental thickness, the maximal uterine flow and the decrease of certain efflux transporters such as P-glycoprotein at term (Ceckova-Novotna et al., 2006; Joshi et al., 2016), the materno-fetal placental transfer rate at term may be considered as higher than at earlier gestational ages (Syme et al., 2004).

The placental transfer process may depend on the physicochemical and molecular properties of BPs, such as molecular size, lipophilicity (LogP), degree of ionization (pKa) and hydrogen-binding capacity (Syme et al., 2004, Gedeon and Koren, 2006, Giaginis et al. 2009). In general, neutral compounds with a molecular weight below 600 Da, high lipid solubility (LogP>1) and few hydrogen bonding sites have a high potential to freely diffuse across the placenta (Audus, 1999; Pacifici and Nottoli, 1995). Thus, the physicochemical properties of these 15 BPs (LogP within 2-7, pKa close to 8.5-9 and MW below 600 Da) would imply that their potential passive diffusion across the placenta would be high (Table 4). However, this is only true for seven BPs (BPAP, BP4-4, BPE, BPF, 3-3BPA, BPA and BPB). By contrast, the restricted transplacental permeability of BPBP, BPZ, BPC, BPM, BPP, BPAF, and, notably of BPS and BPFL, cannot be predicted solely from their physicochemical properties (Table 3).

	Molecular weight (Da)[a]	pKa[a] acidity constant	log P[b] partition coefficient	percent unbound to plasma proteins (Grumetto, 2019)	Clearance indices[c] (mean ± SD)
BPFL	350.13	9.14	5.59		0.065 ± 0.020
BPS	250.03	8.44	2.15	13.23	0.079 ± 0.017
BPBP	352.15	9.21	6.02		0.257 ± 0.046
BPZ	268.15	9.45	5.00		0.318 ± 0.090
BPC	280.01	9.23	3.95		0.392 ± 0.074
BPM	346.19	9.47	7.04	0.98	0.444 ± 0.148
BPP	346.19	9.49	7.04		0.451 ± 0.047
BPAF	336.06	8.90	5.02	1.10	0.522 ± 0.072
BPAP	290.13	9.36	5.00		0.570 ± 0.062
BP4-4	186.07	9.45	2.52		0.663 ± 0.136
BPE	214.10	9.59	3.18	5.82	0.685 ± 0.158
BPF	200.08	9.66	2.91	7.58	0.696 ± 0.144
3-3BPA	256.15	9.74	4.81		0.722 ± 0.070
BPA	228.12	9.52	3.32	4.09	0.813 ± 0.063
BPB	242.13	9.49	4.44	3.21	0.842 ± 0.079

Table 3: Chemical properties of bisphenols and relationship with the clearance index. [a]ChemDraw17.1 (Molecular Networks). [b]ChemDraw17.1 (ChemPropPro). [c]The BP-studied placental transfer rate divided by the antipyrine transfer rate

The maternal plasma protein binding mechanism limits the rate of transfer across the placenta because only the free fraction of a molecule can diffuse across a cell membrane. A recent study to determine the affinities for human serum albumin of bisphenol A and several of its analogues, both by in vitro biomimetic LC and *in silico* prediction, showed that the interaction was largely non-specific and mainly lipophilicity-driven (Grumetto, 2019). The predicted limited unbound fractions of BPM and BPAF (0.98 and 1.10%, respectively) compared to those of highly diffusible BPs (BPE: 5.82%, BPF: 7.58%, BPA: 4.09% and BPB: 3.21 %) are consistent with their differences in placental transfer rate. By contrast, the materno-fetal transfer of BPS is very limited even though its predicted free fraction in serum is relatively high (13.23 %).

Alternatively, the materno-fetal transfer of BPS and BPFL might be restricted by the activity of an efflux transporter. Indeed, several ATP-dependent transporters including multi-drug resistant proteins and organic anion transporter (OAT) proteins, predominantly expressed in the syncytiotrophoblast, are involved in the efflux transport of a wide variety of xenobiotics and thereby in protection of the fetus (Syme et al., 2004). They are known to transport BPs compounds (Jin and Audus, 2005; Mørck et al., 2010) and their levels could be altered by bisphenols (Sieppi et al., 2016; Speidel et al., 2018). However, previous data showed that P-glycoprotein is not involved in limiting the materno-fetal placental transfer of BPS (Grandin et al., 2019). Further investigation is required to see if small structural changes in these analogues could influence the binding sites to transporters and thus the mechanism of transplacental transfer.

4. Conclusion

In conclusion, this is the first study to compare the materno-fetal placental transport of fifteen BPs perfused simultaneously through the term placenta, using the human placental perfusion model. These newly-introduced bisphenols present a wide range of placental transfer rates despite their similar molecular structures. Passive diffusion was the main transport mechanism for seven BPs, BP4-4, BPAP, BPE, BPF, 3-3BPA, BPB, BPA. By contrast, the placenta transfer rates for BPFL and BPS were very limited, and intermediate for BPBP, BPZ, BPC, BPM, BPP and BPAF suggesting that their passage across the placenta does not rely solely on weak diffusional

403 permeability but might involve active transport. However, the placental transfer efficiency of these
404 structurally related bisphenols, determined *ex vivo,* cannot be explained by their physico-chemical
405 properties alone. The data obtained for these fifteen bisphenols will be particularly useful for further
406 development of Quantitative Structure-Activity Relationships (QSARs) models to evaluate the
407 relative contributions of physicochemical, molecular and structural properties in the placental
408 transfer of bisphenols in placental process (Giaginis et al., 2009), and predict the placental
409 transport of this important class of emerging contaminants. This could lead to rapid screening and
410 subsequent discarding of endocrine-active BPA substitutes with a high fetal exposure potential.

411

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649 Figure legends

650 Figure 1: Molecular structures of the fifteen BPs

651 Figure 2: Clearance indices of the 15 BPs (individual values (x) and mean ± SD), corresponding to

652 the ratio of the transfer rate of each bisphenol to that of antipyrine.

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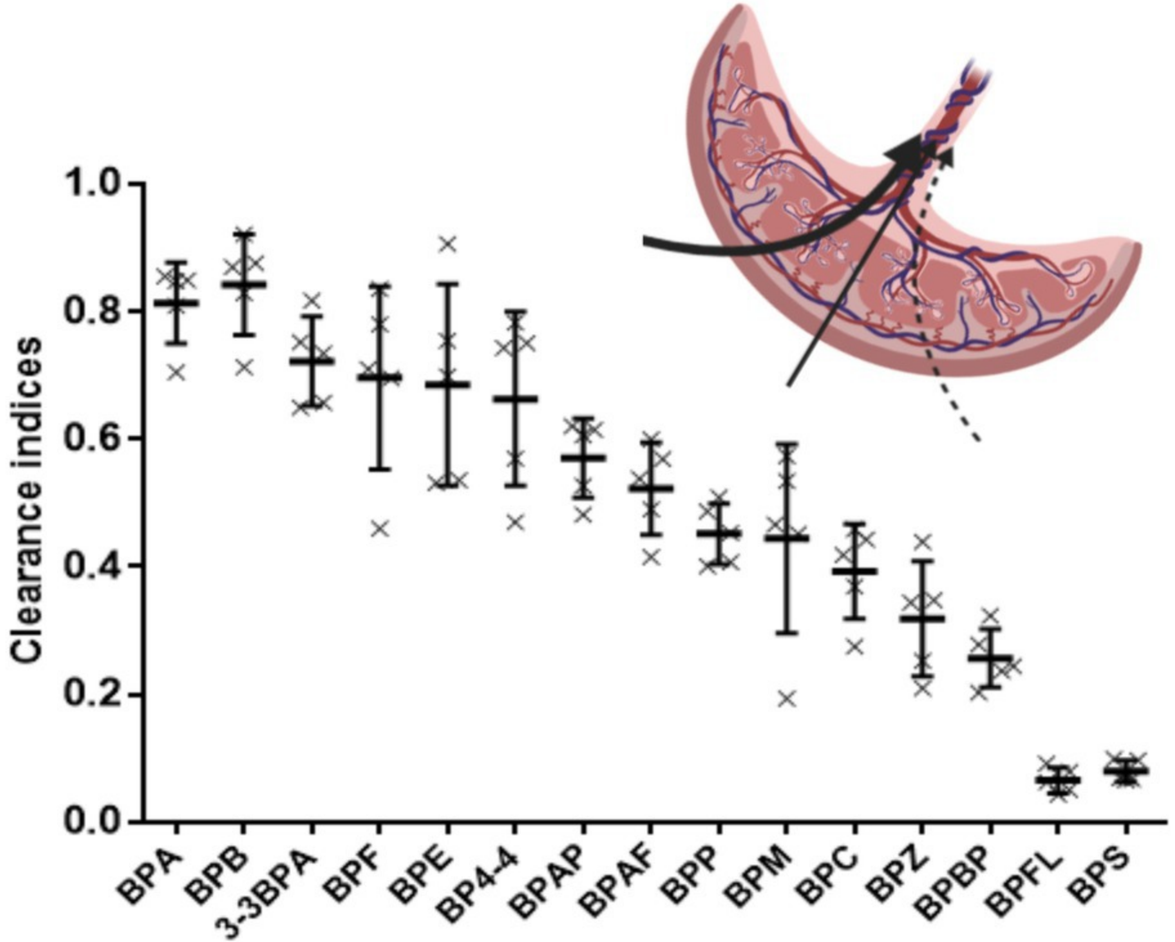

Placental transfer efficiencies