A new LC/MS method for specific determination of human systemic exposure to bisphenol A, F and S through their metabolites: Application to cord blood samples

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Environment International 151 (2021) 106429

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

A new LC/MS method for specific determination of human systemic exposure to bisphenol A, F and S through their metabolites: Application to cord blood samples

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ARTICLE INFO

Handling Editor: Lesa Aylward

Keywords:
Bisphenol analogs
Bisphenol glucuronides
Dansyl chloride
Cord plasma
LC/MS

ABSTRACT

Due to restriction of the use of BPA, several structural analogues such as BPS and BPF have been proposed for its replacement in many consumer products. This has increased the prevalence of BPS and BPF in urine from tested cohorts. However, these substitutes have similar endocrine disrupting properties to BPA, particularly on reproductive and metabolic functions, which suggests that fetal exposure to these analogues could be of concern for human health. Bisphenols (BPs) are mainly metabolized to glucuronides (BP-Gs), which are considered as inactive but provide a relevant marker of fetal exposure during pregnancy. In most instances, these metabolites are indirectly quantified after hydrolysis and measurement of the corresponding native BPs, which may lead to bias due to spurious BPs contamination during blood collection and/or analyses. We have developed a new method for direct quantification of BP-Gs, which has the advantage of not being affected by errors related to the presence of BPs. First, BP-Gs were extracted from plasma by anion exchange solid phase extraction. They were then labelled with dansyl chloride, using experimentally-optimized incubation conditions, after which the dansyl derivatives were injected into an on-line SPE-UHPLC/MS/MS system. The performance of the method, in terms of sensitivity, precision and accuracy, was evaluated in plasma over a concentration range of 0.05–5 ng/mL. The intra- and inter-day CV% precision were lower than 20% with accuracies ranging from 93% to 115%. The limit of quantification was set at 0.05 ng/mL. The method was then applied to measure BP-Gs in forty-four cord plasma samples. Although no BPF-G was found, BPA-G and BPS-G was determined in almost half of the cord plasma samples with concentration ranges nd-0.089 ng/mL and nd-0.586 ng/mL, respectively.

1. Introduction

Due to restrictions of its use, bisphenol A (BPA) has gradually been replaced by structural analogues such as bisphenol S (BPS) and bisphenol F (BPF) in many consumer products. The extent of this substitution has been revealed by the high prevalence of BPS and BPF in urine from cohorts of pregnant women (Derakhshan et al., 2019; Hu et al., 2019; Jiang et al., 2020), thus raising the question of the risk of fetal exposure to these endocrine-active compounds. Indeed, *in vitro* studies have shown that the potencies of the estrogenic activities displayed by BPS and BPF are of the same order of magnitude as those of BPA (Pelch et al., 2019; Rochester and Bolden, 2015). Animal studies of the developmental effects of exposure to these bisphenols (BPs) on functions sensitive to BPA disruption, such as reproduction and metabolism, suggest that fetal exposure to these analogues could be of concern for human health (see Pelch et al. 2019 for review). BPs are mainly metabolized to glucuronides (see Gramec Skledar et Peterlin Mašič 2016 for review). Although BPs glucuronides (BP-Gs) are considered as inactive metabolites, *in vitro* studies have suggested that BPA-G can exert biological activities (Boucher et al., 2015; Viñas et al.,

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https://doi.org/10.1016/j.envint.2021.106429
Received 29 September 2020; Received in revised form 20 January 2021; Accepted 27 January 2021
Available online 23 February 2021

2013). Since some of the related effects may result from possible deconjugation of BPA-G (Gayrard et al., 2015), these observations highlight the added value of quantifying both the plasma concentrations of parent BPs and their metabolites during human biomonitoring studies.

Previous toxicokinetic (TK) studies undertaken in pregnant ewes have shown that BPA, BPS and BPF can cross the placenta (Corbel et al., 2013; Grandin et al. 2018; Gingrich et al. 2019). Although the maternal-to-fetal transfer of BPS in ewes (0.4% of the dose, Grandin et al. 2018), was lower than that of BPA (6%, Corbel et al., 2013; Gauderat et al., 2016), the predicted fetal plasma concentrations of unconjugated BPS were similar to those of BPA due to the slow elimination of BPS from the fetal compartment (Grandin et al. 2018). The *ex vivo* model of human perfused placenta showed that the human maternal-to-fetal placental clearance of BPS was of the same order of magnitude as that estimated in sheep experiments and that BPS-G cannot cross the placenta (Grandin et al. 2019). These various results indicate that BPS-G in fetal blood is mainly of fetoplacental origin and, like BPA-G, may represent a relevant marker of fetal exposure to BPs (Gauderat et al., 2017, 2016). While a large number of studies have assessed BPA concentrations in cord blood, currently, only three assessments of BPA analogues in human cord blood have been carried out one from direct measurement of unconjugated BPS and BPF (Pan et al., 2020) and the other two from indirect measurement of total BPS (unconjugated and conjugated) using enzymatic hydrolysis (Liu et al., 2017b; Zhang et al., 2020). However, there is concern that evaluations of BPs concentration could be biased due to external contamination of the samples during blood collection and/or analyses (Ye et al., 2013). In contrast, direct measurement of BP-Gs has the advantage of not being affected by this kind of bias due to the absence of BP-Gs in the environment.

Direct analysis of BP-Gs presents a challenge because the glucuronide moieties make these compounds difficult to extract from biological matrices, retained in liquid chromatography and efficiently ionize in mass spectrometry. All of the few methods developed to assay BP-Gs in biological matrices have simultaneously quantified BPA and its metabolites (Andra et al., 2016; Gerona et al., 2016; Ho et al., 2017; Lacroix et al., 2011; Provencher et al., 2014; Völkel et al., 2005) and those of BPS and BPS-G (Grandin et al. 2017). Some of these methods, intended for application in toxicokinetic studies, are not sensitive enough to assess the levels of residual human exposure to BPs (Grandin et al., 2017; Lacroix et al., 2011; Völkel et al., 2005). To increase the sensitivity of BPs detection by mass spectrometry, several methods involved derivatization with dansyl chloride or its analogues (Li and Franke, 2015; Regueiro et al., 2015; Vitku et al., 2015). These reagents have been shown to improve selectivity and the level of detection by switching negative electrospray ionization (ESI-) to positive electrospray ionization (ESI +) and increasing the *m/z* ratio. In a previous study, these derivatization methods were already described for human biomonitoring BPA-G and BPS-G in urine (Rancière et al., 2019) but they have never been applied to assay BP-Gs in human plasma, which required higher sensitivity.

In this context, the objective of the present study was to develop a reliable method for quantifying the glucuronoconjugated metabolites of BPA and its major substitutes, BPS and BPF (BPA-G, BPS-G and BPF-G) in human plasma according to the European Medicines Agency guidelines on bioanalytical method validation (European Medicines Agency, 2009). To achieve this, the derivatization reactions with chloride dansyl and the on-line solid phase extraction (SPE) procedures were first optimized using experimental designs. The performance of the method was assessed by measuring BP-Gs concentrations in human cord plasma samples.

2. Material and method

2.1. Chemicals

Acetonitrile (AcN) and methanol (MeOH) were LC/MS quality and purchased from Fisher Scientific (Illkirch, France). Formic acid 98% (HCOOH) was purchased from Fluka Analytical (St Louis, Missouri, USA), sodium bicarbonate was purchased from Fisher BioReagents (Gell, Belgium) and water was obtained from an ultrapure water system (PureLab Classic, Veolia Water). Dansyl chloride (DnCl), 1-methylimidazole-2-sulfonyl chloride (ISCl), pyridine-3-sulfonyl chloride (PsCl), ammonium acetate > 99.9%, ammonium hydroxide > 99.99% and sodium carbonate were purchased from Merck (Darmstadt, Germany). Bisphenol A mono β-D-glucuronide (BPA-G), bisphenol F mono β-D-glucuronide (BPF-G) and bisphenol S mono β-D-glucuronide sodium salt (BPS-G), used as analytical standards, and bisphenol S mono β-D-glucuronide d8 sodium salt (BPS-Gd_8) and bisphenol A $^{13}C_{12}$ (mono β-D-glucuronide) (BPA-G $^{13}C_{12}$) used as internal standards (IS) were provided by Toronto Research Chemicals (TRC, Toronto, Canada).

2.2. Instruments and analytical conditions

2.2.1. Instruments

BPA-G, BPS-G and BPF-G were identified and quantified using a 2D Acquity ultra performance liquid chromatography system (2D-UPLC®) coupled to a Xevo® triple quadrupole mass spectrometer (Waters®, Milford, MA, USA). The two UHPLC pump were connected with the autosampler and the mass spectrometer by two 6-port valves integrated in the column heater (Fig. 1). The first of the two UHPLC pumps in the chromatographic system was used for extraction with on-line SPE and the second pump for analysis. BPA-G, BPS-G and BPF-G were first loaded on the XBridge® C8 Direct Connect HP cartridge (2.1x30mm, 10 µm) then separated on an Acquity UPLC® CSH™ Phenyl-Hexyl column (2.1x100mm, 1.7 µm), with both columns set at 40 °C. Samples were ionized in ESI positive ion mode. The capillary voltage and source temperature were set at 3.5 kV and 150 °C, respectively. The desolvation temperature and nitrogen flow rate were set at 600 °C and 800 L/Hr,

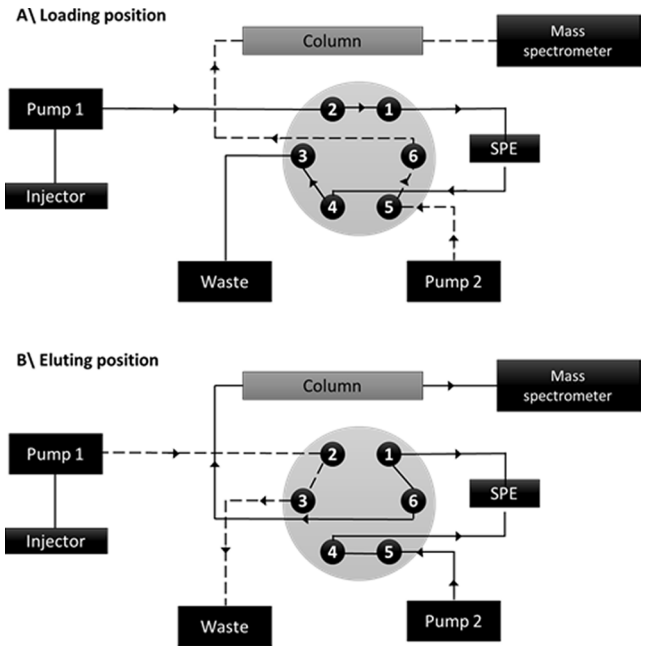

Fig. 1. Configuration of the on-line SPE UHPLC/MS/MS system. A/ Loading position: the pump 1 eluted the SPE cartridge at 1 mL/min. The pump 2 equilibrated the analytical column. B/ Eluting position: the pump 2 eluted both the SPE cartridge and the analytical column.

respectively. Argon was used as the collision gas.

2.2.2. UHPLC-MS-MS analysis

The solvent used for the on-line SPE and the analytical pump were H_2O acidified with 0.1% HCOOH (%A) and AcN acidified with 0.1% HCOOH (%B). The BPA-G, BPS-G and BPF-G were pre-concentrated on the C8 Cartridge under the following conditions : t(0 → 0.5 min) 80%A, 1 mL/min flow rate; t(0.5 → 0.6 min) 0%A , 0.1 mL/min ; t(0.6 → 8 min) 0 %A, 1 mL/min; t(8 → 9 min) 80%A, 1.0 mL/min; t(9 → 10 min) 80%A, 1.0 mL/min. The BP-Gs were eluted on a Phenyl-Hexyl column at 0.35 mL/min using a linear gradient: t(0 → 0.5 min) 70%A ; t(0.5 → 9 min) 70% A → 5 %A; t(9.0 → 9.1 min) 70%A. The valves switched from loading to eluting position at t = 0.5 min and went back to their initial position at t = 5 min (Fig. 1). The BPA-G, BPS-G and BPF-G and IS were detected in ESI^+ by multiple reaction monitoring (MRM) mode. MRM transitions of analytes and IS with cone voltages and collision energies are given in Table 1. Chromatographic data were monitored by Masslynx 4.1® software (Waters®, Milford, MA, USA).

2.3. Preparation of solution

Stock solution were prepared for each BP-G by dissolving 1 mg of powder in 1 mL AcN/H_2O (50/50). Working solutions were prepared by serial dilution of BPA-G, BPS-G and BPF-G in AcN/H_2O (50/50) to obtain final concentrations of 100 μg/mL, 1000, 100 and 20 ng/mL of each analyte. Standard and quality control (QC) solutions were obtained by diluting working solutions in BPs-free human plasma to obtain concentrations ranging from 0.05 to 5.0 ng/mL for the standard curve calibrator and at 0.15 to 0.375 and 3.75 ng/mL for QC samples. The solution of internal standard (IS, BPS-Gd_8, BPA-G $^{13}C_{12}$) was prepared in H_2O/MeOH: 50/50 at 50 ng/mL and stored at 4 °C.

2.4. Sample preparation

2.4.1. Extraction procedure

BP-Gs were extracted from the matrices with HR-XAW cartridges (Marcherey Nagel, Düren, Germany). The samples (200 μL) and 50 μL of IS (BPS-Gd8, BPA-G $^{13}C_{12}$ 50 ng/mL) were loaded on the SPE cartridges previously conditioned with 1 mL MeOH and 1 mL ultrapure water. The sorbent was rinsed with 1 mL of 25 mM ammonium acetate ($AcONH_4$) and 1 mL MeOH. The cartridges were dried for 5 min and the analytes were eluted with 1 mL MeOH, 1% NH_3. The elution solutions were evaporated to dryness at 45 °C under nitrogen flow.

2.4.2. Derivatization

The extracts were reconstituted in 100 μL HCO_3 buffer (pH 7.5, 250 mM) and derivatized after addition of 100 μL dansyl chloride (1 mg/mL in AcN) at 38 °C, 500 rpm and for 45 min. The solution was centrifuged at 4 °C and 20000g for 10 min and acidified with 0.2 μL of HCOOH. One hundred microliters of supernatant were collected in 300-μL LC/MS quality glass inserts adapted for 2 mL-vials screwed with teflon caps (Macherey Nagel).

Table 1
MRM transitions of BP-Gs derivatized with DnCl with their respective cone voltages, collision energies and retention times (RT).

	Dn-BPA-G	Dn-BPF-G	Dn-BPA-G$^{13}C_{12}$	Dn-BPS-G	Dn-BPS-Gd_8
m/z Precursor ion	638	610	650	660	668
Cone voltage (V)	26	30	26	32	32
Product ion 1[a]	462	434	474	484	492
Ecoll (eV)	24	24	24	24	26
Product ion 2	171	171	171	171	171
Ecoll (eV)	46	48	46	48	48
RT (min)	5.5	5.0	5.5	4.6	4.6

[a] MRM transition with product ion 1 was used for quantification.

2.5. Optimization

2.5.1. Derivatization process

2.5.1.1. General set up. Three reagents were tested for derivatization: Dansyl chloride (DnCl), 1-methylimidazole-2-sulfonyl chloride (ISCl), and pyridine-3-sulfonyl chloride (PSCl). Dansyl chloride was retained for the subsequent phase of optimization. BP-Gs were diluted to 20 ng/mL in H_2O/AcN (50/50) in order to ensure sufficient detection sensitivity for each condition of the experimental design. BP-Gs (100 μL) were derivatized with DnCl (1 mg/mL in AcN) in 1.5 mL microtubes by adding 100 μL/200 μL of HCO_3 buffer (pH 7.5–11) and 200 μL/100 μL of DnCl. The volume of the buffer was adjusted in order to keep the same final volume for the different tests on the ratios of the volume between DnCl and the sample (V_{DnCl}/V_{sample}).The microtubes were heated from 25 °C to 60 °C and stirred at 500 rpm (Mixing Block MB-102 BIOER) for 5 to 90 min. The resulting mixture was centrifuged at 4 °C and 20,000 g for 10 min (Centrifuge 5417R Eppendorf). Twenty microliters of each derivatized solution were injected into the on-line SPE-UHPLC/MS/MS system.

2.5.1.2. Experimental design. The response to be optimized was the peak area, using the factors of incubation temperature, reaction time, buffer pH and ratio of the volume between DnCl and the sample (V_{DnCl}/V_{sample}). Firstly, the effect of each factor and their interactions were studied with a Hadamard matrix built with two levels and four factors, which lead to carry out sixteen experiments. The first experiment was repeated five times to estimate the experimental variability in order to assess the statistical significance of the effect of each factor and their interactions through an Analysis of Variance model (ANOVA, $p < 0.1$). Then, a Doehlert design was performed to model the evolution of BPS-G, BPA-G and BPF-G peak area as a function of buffer pH and incubation temperature (Supplemental data S1).

2.5.1.3. Reaction kinetics. The derivatization reaction was monitored for 90 min under the optimized conditions (i.e. pH 7.5 and T = 38 °C) with a solution of BPA-G, BPF-G and BPS-G at 20 ng/mL to determine the optimal reaction time. The solutions were collected every 5 min and analyzed by UHPLC/MS/MS.

2.5.2. On-line SPE pre-concentration

Three commercially available cartridges were tested: C8 (XBridge® C8 Direct connect HP 2,1 × 30 mm, 10 μm, Waters), C18 (XBridge ® C18 Direct connect HP 2,1 × 30 mm, 10 μm, Waters) and HRX (Chromabond 15 μm, Marcherey Nagel, Düren, Germany). Charge time and H_2O initial percent value were optimized using the Doehlert experiment design for each cartridge (Supplemental data S3).

2.6. Performance of the method

The performance of the method was checked in terms of linearity, inter- and intra-day precision and accuracy, and sensitivity (European Medicines Agency, 2009). Limit of blank (LOB) was evaluated by measuring 8 replicates of a blank sample and calculating the mean peak area (without IS) and the standard deviation (SD) with the following equation LOB = mean blank + 1.645*SD(blank). The LOD (limit of detection) was calculated according to the following equation LOD = 3.3*SD(LLOQ)/S. SD(LLOQ) was the standard deviation of the response at the lower limit of quantification (LLOQ). S was the slope of a line drawn between the origin and the mean response of the eight replicates at LLOQ, the response being the peak area ratio of the analyte to IS. The LLOQ was evaluated with eight replicates of plasma samples spiked at

0.05 ng/mL and was set as the lowest concentration level of the calibration curve that could be quantified with acceptable repeatability and accuracy. Seven calibration points containing the three BP-Gs at concentrations ranging from 0.05 to 5.0 ng/mL were extracted and assayed over three days. Both linear ($Y = aX + b$) and quadratic ($Y = aX^2 + bX + c$) models were tested with 1, 1/X and $1/X^2$ (X = nominal concentration) weightings with these resulting calibration curves. Three approaches were tested to select the best calibration model: 1) inspection of the residual distribution plot against nominal concentrations, 2) a lack-of-fit test to check the goodness-of-fit of the model and 3) calculation of the relative concentration residuals (RCR%) between the nominal concentration and the concentration obtained with the model, which should be lower than ± 15%. Intra-day and Inter-day precisions and accuracies were calculated on three different days and with five replicates of QC samples at three concentration levels (Low = 0.15 ng/mL, Medium 0.375 ng/mL and High = 3.75 ng/mL) covering the range of the calibration curve concentrations (0.05–5 ng/mL). Intra and inter-day precisions were expressed with a coefficient of variation percent (CV% = 100* Standard Deviation (SD)/mean). The intra- and inter-day standard deviations were calculated using an one-way ANOVA (Eqs. (1) and (2)).

$$Intra - daySD = \sqrt{WMS} \quad (1)$$

$$Intra - daySD = \sqrt{\frac{BMS - WMS}{n} + WMS} \quad (2)$$

with: WMS = within group mean square
BMS = Between group mean square
n = number of observation

The stability of the BP-Gs derivatives in the autosampler was assessed by reanalyzing the QC samples stored at 15 °C and in darkness over time. A first set of three QC samples at low and high concentrations were injected at t0, 10 and 12 h, a second set at t2, 4 and 6 h and a third set were injected at t0 and 24 h. Each concentration was compared to its nominal value and a paired *t*-test was performed on each set and between time to check the stability of QC concentration over time. Matrix effects and SPE recovery were evaluated in triplicate by comparing the MRM responses of extracted blank plasma spiked with 5 ng/mL of BP-Gs and IS after SPE extraction to solvent spiked with 5 ng/mL of BP-Gs and IS and extracted plasma spiked with 5 ng/mL before SPE. Carry-over was assessed by injecting three solvent samples after the high QC level and comparing the resulting BP-Gs area to the BP-Gs area obtained at the LLOQ.

2.7. Routine analysis and validation

Each week, the first analytical run consisted of the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS), the seven calibration standards and 3 levels of QC samples (low, medium and high). The calibration curve was validated if at least 75% of the calibration standards had back calculated concentrations within ±15% of the nominal value, except for the LLOQ for which it could be within ±20%. Each day, an initial set of QC samples was assayed to validate the weekly calibration curve, after which approximately 80 samples per day were extracted and labelled. The results as a whole were validated with the second set of QC added at the end of the daily analysis and according to the dansylation stability. The daily analysis was validated if at least 67% of the QC samples and at least 50% at each concentration level were within ± 20% of their nominal values (European Medicines Agency, 2009).

2.8. Blood sampling

Forty-four pregnant women were recruited at the gynaecology-obstetric unit of the Centre Hospitalier Universitaire (CHU) of Toulouse (France) between June 2014 and October 2015, according to the standard guidelines set by the ethical committee of the CHU. The study received institutional approval (DC-2013–1950) and participants provided informed consent for data collection. The cord blood was collected with heparinized vacutainer tubes in Polyethylene Terephthalate (BD vacutainer 367869). Samples were centrifuged for 10 min at 3000 g. The resulting plasma was stored at −20 °C in polypropylene tubes until assay.

3. Results and discussion

3.1. Derivatization

In general, BPs are poorly ionizable in ESI under the pH ranges necessary for robust chromatographic retention and chemical derivatization is often used to enhance ionization efficiency. Indeed, the first derivatization procedure described for the quantification of BPs in biological fluids involved DnCl; the sulfonyl group of DnCl readily reacts with the phenolic functions of BP and leads to a dansyl derivative that is highly ionizable in positive mode (Chang et al., 2010). For this reason, other analogues of DnCl such as ISCl and PSCl were also tested to enhance BPs ionization and improved sensitivity by a factor of 9 to 50 (Li and Franke, 2015; Regueiro et al., 2015; Vitku et al., 2015). However, these derivatizing agents have never been compared on glucuronide metabolites, to our knowledge, so we tested them on the three BP-Gs. Since only one phenolic function is available on BP-G, only one derivatizing agent can react on BP-G, unlike BP which can be derivatized twice (Fig. 2) (Li and Franke, 2015; Wang et al. 2018; Regueiro et al., 2015; Vitku et al. 2015)

In mass spectrometry, the amine function present on the derivative portion enabled all the analytes to produce the $[M + H]^+$ parent ions. The three BP-Gs were derivatized with the three derivatizing agent in the conditions reported for BPs in the literature, i.e. 15 min of incubation at 60 °C and pH 9 (Chang et al., 2010, Li and Franke, 2015; Regueiro et al., 2015; Vitku et al., 2015). By comparing the three derivatizing agents with each other, DnCl derivatization was shown to produce a signal 5 and 10 times higher than ISCl and PSCl, respectively. Moreover, the best chromatographic results, i.e. the higher peak area with a good peak shape, were obtained with DnCl on the phenyl-hexyl column and under acidic conditions (Fig. 3). Therefore, the method for quantification of BP-Gs was optimized using DnCl as derivatizing agent. The MRM transition corresponding to the neutral loss of glucuronic acid had both the highest intensity and best selectivity for all the three analytes and was thus chosen as quantifier ion. The second MRM transition, which corresponded to the R- fragment of the dansyl chloride was used for confirmation (Supplemental data S2)

3.2. Optimization of the condition of dansylation by experimental design

Experimental designs are often used in biological, environmental, and pharmaceutical applications to optimize conditions in the shortest time both by reducing the number of experiments and taking interactive effects into account (Hanrahan and Lu, 2006). Thus, a full factorial design and a Doehlert design was used to optimize the dansylation of BP-Gs.

3.2.0.1. Full factorial design

A two-level full factorial design was first carried out to determine the main factors influencing dansylation and their interactions. The pH bounds (9 and 11), the ratio of the volume between DnCl and sample (V_{DnCl}/V_{sample}; 1/1 and 2/1), the incubation time (5 min and 30 min) and the temperature (50 °C and 60 °C) were selected from those frequently described for BPs dansylation (Wang et al., 2018). The most influential parameters were V_{DnCl}/V_{sample}, pH and temperature and their interactions but no single combination gave the best response for all three BP-Gs. Thus, a Doehlert experimental design was performed to refine the parameters of each BP-G in order to determine the parameters

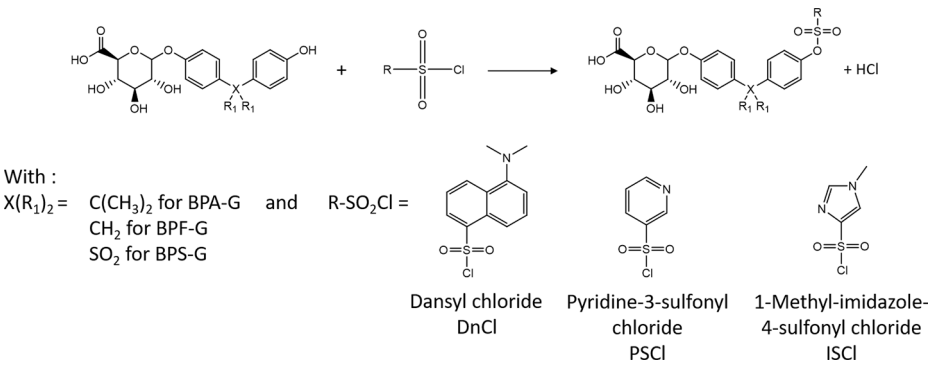

Fig. 2. Derivatization of the three BP-Gs with Dansyl chloride (DnCl), Pyridine-3-sulfonyl chloride (PSCl) and 1-Methyl-imidazole-4-sulfonyl chloride (ISCl).

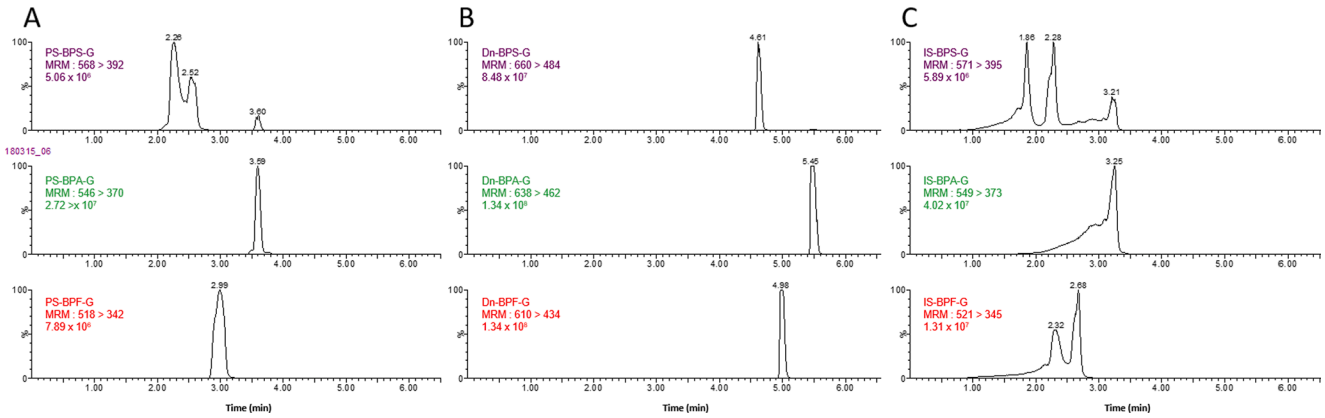

Fig. 3. MRM chromatograms of the three BP-Gs derivatized with A) Pyridine-3-sulfonyl chloride (PSCl), B) Dansyl chloride (DnCl) and C) 1-Methyl-imidazole-4-sulfonyl chloride (ISCl).

common to the three BP-Gs.

3.2.0.2. Doehlert experimental design

An initial Doehlert design was built by setting the V_{DnCl}/V_{sample} ratio at 1/1 and using the central point previously determined with the full

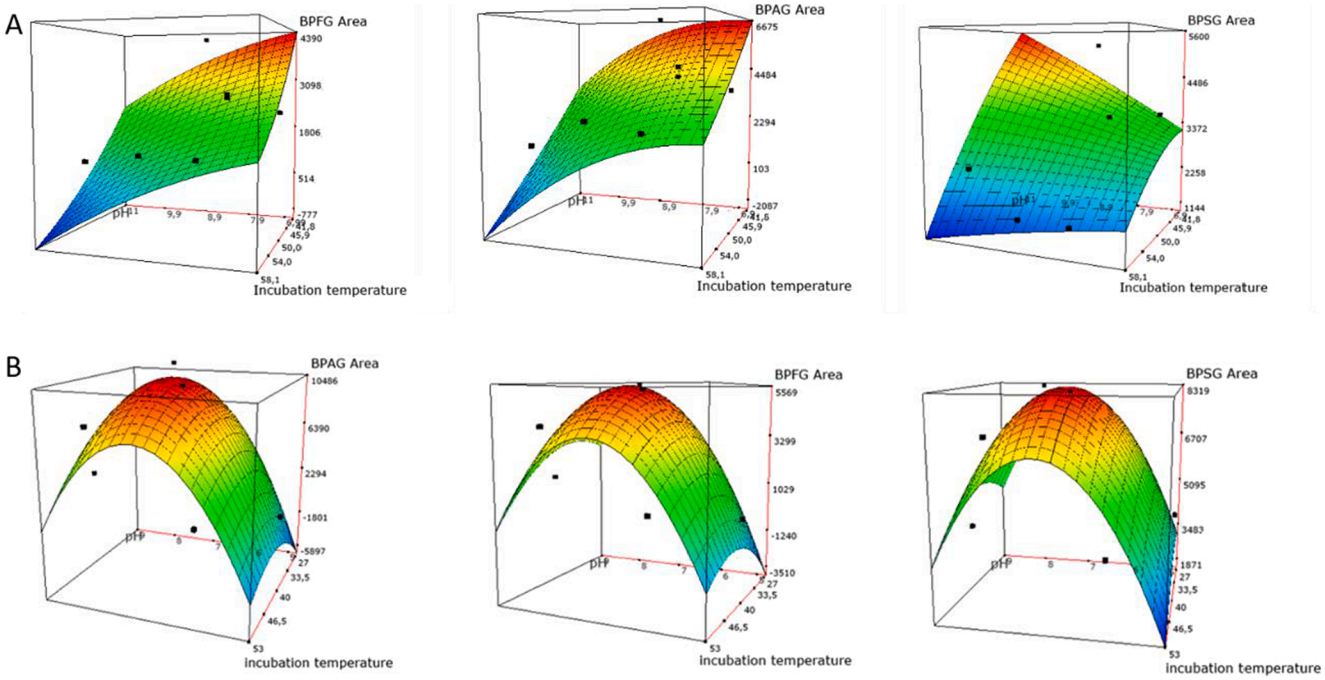

Fig. 4. MRM response of Dn-BPA-G, Dn-BPF-G and Dn-BPS-G as function of the incubation temperature and pH obtained with an initial Doelhert design centred at pH9 and T50°C (A) and with a second Doelhert design shifted to pH7 and T40°C (B).

factorial design (pH9, t30 min and T50°C). However, the maximum responses were systematically observed at the end of the experimental region, which meant that the bounds were not correctly defined for either temperature or pH (Fig. 4A). The center of the Doehlert design was therefore shifted to a lower temperature (40 °C; bound 27–53 °C) and a lower pH (7; bound pH6-9).

As shown in Fig. 4B, the dansylation of BP-Gs is mainly dependent on pH. The optimal conditions for Dn-BP-Gs are milder than for Dn-BPs (pH 9 and T°C = 60 °C). One explanation for these results could be possible glucuronide hydrolysis, which can occur in this mixture of solvents and at high temperatures (Jr et al., 2007). To ensure that no hydrolysis occurred during incubation, MRM transitions of BPs were monitored after BP-Gs dansylation both under the optimized conditions (pH7.5, 38 °C) and the harshest conditions described for BPs (pH 11 and 60 °C). No hydrolysis of BP-Gs was observed under either of the dansylation conditions. Subsequently, the time of incubation was evaluated by monitoring the response of Dn-BP-Gs formation every 5 min for 90 min. The formation of Dn-BPS-G was faster and more stable than that of Dn-BPA-G and Dn-BPF-G. Indeed, Dn-BPS-G was completely synthesized within 15 min while complete dansylation of BPA-G and BPF-G occurred after 40 min of reaction. The optimal incubation time obtained under these conditions was longer than under the usual conditions adopted for BPs (5–10 min). However, these incubation conditions made the derivatives more stable and their MS responses started to decline after 75–85 min of incubation whereas they decreased after 20–30 min under the conditions used for BPs (Wang et al., 2018).

3.3. Solid phase extraction

3.3.1. On-line SPE

After derivatization with DnCl, samples were loaded on the on-line solid phase extraction system coupled to the UHPLC/MS/MS in order to increase the BPs-G response. Three cartridges were tested and the SPE conditions (load time and solvent percentage) were optimized for each cartridge. The best results, i.e. the best signal-to-noise ratio and the highest peak area, were obtained with a C8 cartridge by loading sample for 0.5 min with 80% H_2O acidified with 0.1% formic acid (Supplementary data S.3.2). Despite improvement of the signal-to-noise ratio, the use of on-line SPE alone was not sufficient enough to obtain a good response of BPs-G at low concentrations: a more specific SPE step was therefore added to improve method sensitivity.

3.3.2. SPE extraction of BP-Gs before dansylation

Two strategies, involving extraction of BP-Gs either after or before dansylation, were considered. Indeed, as Dn-BP-Gs compounds are larger and less polar than BP-Gs, they should be easier to extract on the usual SPE cartridges (C8, C18…). However, these dansylated compounds might be degraded during the different extraction steps of the SPE. Inversely, BP-Gs extraction before dansylation might prove more difficult, due to the polarity of the glucuronides, even though anionic exchange SPE have been developed for these kinds of molecules(Provencher et al., 2014). Such SPE facilitate hydrophobic interactions with the aromatic rings of BP and also promote exchanges with the glucuronic acid function. In addition, the reaction with DnCl might be more effective if the BP-Gs were first extracted from the matrices, i.e. before dansylation, as this would reduce competitive reactions with any interferences. Firstly, BP-Gs extractions were developed on HR-XAW cartridges before dansylation using plasma spiked at 100 ng/mL. Retention of the BP-Gs on the cartridges was determined by direct assay of the wash solution (25 mM $AcONH_4$) and the two elution steps (MeOH and MeOH, 1% NH_3) were directly assayed on UHPLC/MS/MS without labelling. Neither BPA-G nor BPF-G were detected during the washing step or during the first elution and were entirely eluted during the second elution step. BPS-G behaved slightly differently: a small quantity (<10%) was detected during the washing step but not in the first elution and it was mainly eluted with MeOH, 1% NH_3. An additional elution (MeOH, 1% NH_3) was tested but did not improve BP-Gs extraction. Blank plasma spiked with IS was subjected to the same SPE protocol, spiked with 5 ng/mL BP-Gs either before or after SPE, and then dansylated. The SPE recovery, i.e. the ratio of IS-normalized peak areas of plasma spiked with BP-Gs before and after SPE, was around 60% for all three BP-Gs. Since stable isotope-labeled BPF-G is not available as analytical standard, and because the physicochemical properties and the SPE recovery of BPA-G and BPF-G are similar, BPA-G$^{13}C_{12}$ was used as the internal standard for both molecules. Matrix factor, i.e. the ratio of the MRM responses of extracted blank plasma to solvent, both spiked with 5 ng/mL of Dn-BP-Gs and IS after SPE extraction was normalized by the MRM responses of the IS (Table 2). IS-normalized matrix factor were close to 100%, for all three Dn-BP-Gs (Table 2).

In conclusion, the total IS-normalized recovery, i.e. the ratio of the Analyte/IS MRM responses of plasma spiked with BPs-G before SPE to the MRM responses in the solvent and at the same concentration levels in solvent, is quite low (55%). This is mainly due to the relatively low SPE recovery, but allows significant increase of the signal-to-noise ratio and improvement of method sensitivity.

3.4. Performance of the method

3.4.1. Calibration curves

Seven calibration points containing the three BP-Gs at concentrations ranging from 0.05 to 5.0 ng/mL were extracted over three days with BPS-Gd_8 and BPA-G$^{13}C_{12}$ as internal standards. The best calibration fit was obtained with a linear model weighted by $1/X^2$ for all three BP-Gs with RCR% lower than 12% for all concentrations and the linear model was confirmed by the lack-of-fit test which was not significant ($P > 0.05$). Then, three plasmatic calibration curves were compared with three aqueous calibration curves at the same concentration range. The Analyte/IS area ratios between plasmatic and aqueous calibration standards varied from 0.9 to 1.26 for BPA-G and BPS-G. For BPF-G, at low concentration levels (0.05 and 0.1 ng/mL), the response in plasmatic calibration curve could be nearby twice higher than in aqueous calibration curve (Supplemental data S4). These results confirmed the need to prepare the calibration standards in the same matrix as the unknown samples in order to avoid bias in the concentration results. Three additional concentrations (10, 20 and 50 ng/mL) were investigated to ensure the efficiency of the derivatization in both plasma and aqueous matrices. On the one hand, the responses were proportional to the concentrations regardless of the matrix for the three Dn-BP-Gs and on the other hand, the response between the two matrices were not different (Supplemental data S4). Thus, since DnCl was added in excess (1 mg/mL), the derivatization was considered to be total in the concentration range of the study.

3.4.2. Limit of detection and limit of quantification

Limit of detection was estimated at 0.02 ng/mL for BPA-G and BPF-G and at 0.03 ng/mL for BPS-G by using the slope between zero and LOQ and the SD at LOQ. The higher estimation the LOD of BPS-G was due to the high responses of BPS-G observed in the blank plasma. Therefore, the limit of blank (LOB) values were determined for each of the three BP-Gs and are provided in Table 3 (Armbruster and Pry, 2008). These results were helpful to set the first concentration of the calibration curve, which was also considered as the LOQ. Contrary to most studies in which the LOQ is determined from the signal-to-noise ratio, our LOQ was

Table 2
IS-normalized matrix factor, SPE and total IS-normalized recovery of BP-Gs obtained in human plasma.

Extraction parameters	Dn-BPA-G	Dn-BPS-G	Dn-BPF-G
IS-normalized-matrix factor (n = 3)	102%	100%	99%
SPE Recovery % (n = 3)	58%	58%	60%
Total IS-normalized Recovery % (n = 3)	55%	54%	55%

Table 3
Performance of the method for quantifying Dn-BPA-G, Dn-BPS-G and Dn-BPF-G in human plasma.

Nominal concentration (ng/mL)		Dn-BPA-G				Dn-BPF-G				Dn-BPS-G			
		Mean (ng/mL)	Mean accuracy (range) %	Precision (CV%)		Mean (ng/mL)	Mean accuracy (range) %	Precision (CV%)		Mean (ng/mL)	Mean accuracy (range) %	Precision (CV%)	
				Intra-day (n = 15)	Inter-day (n = 3)			Intra-day (n = 15)	Inter-day (n = 3)			Intra-day (n = 15)	Inter-day (n = 3)
LOB (n = 8)		0.016				0.005				0.041			
LOD[a] (n = 8)		0.025				0.023				0.038			
LOQ (n = 8)	0.05	0.046	92%	30%		0.051	101%	26%		0.052	103%	22%	
QC (n = 5 × 3)													
Low	0.15	0.163	109% (104–115%)	18%	18%	0.139	93% (80–101%)	17%	20%	0.167	111% (108–116%)	13%	13%
Mid	0.375	0.430	115% (104–121%)	8%	11%	0.377	100% (97–104%)	5%	6%	0.393	105% (104–111%)	8%	9%
High	3.75	3.988	106% (102–110%)	3%	4%	3.945	105% (100–109%)	2%	5%	3.847	103% (99–105%)	3%	4%
Stability[b]													
Low QC (n = 3)													
10*H*	0.15	0.189	126%	13%		0.129	86%	20%		0.192	128%	11%	
12H	0.15	0.163	109%	27%		0.115	77%	4%		0.150	100%	12%	
24H	0.15	0.131	87%	6%		0.076	51%	42%		0.162	108%	17%	
High QC (n = 3)													
10*H*	3.75	3.842	102%	1%		3.582	96%	1%		3.728	99%	2%	
12H	3.75	3.763	100%	4%		3.586	96%	9%		3.818	102%	3%	
24H	3.75	3.991	106%	4%		3.675	98%	4%		3.988	106%	3%	

[a] LOD evaluated with the slope between zero and LOQ and the SD at LOQ: LOD = 3.3*SD(LOQ)/Slope.
[b] Autosampler stability evaluated in the darkness and at 15 °C.

considered as the lowest concentration that could be quantified with acceptable accuracy and precision (European Medicines Agency, 2009). Eight replicates at 0.05 ng/mL (0.1 nM) were assayed over one day to determine LOQ performance for the three BP-Gs. Accuracy ranged between 92% and 103% and the intra-day precision was lower than 30% (Table 3). On the one hand, these LOQs are consistent with those reported for the direct measurement of BPs in plasma (BPS : 0.04 nM ; BPA : 0.8 nM, estimated with the signal-to-noise approach, Zhang et al., 2020; and BPA: 0.16 nM measured from standard deviation low concentration samples, Yamamoto et al., 2016). On the other hand, they are also consistent with those obtained with dansylation of native BPs (BPA: 0.04 ng/mL, i.e. 0.16 nM, Vitku et al., 2015).

3.4.3. Precision and accuracy

Intra-day and inter-day precision were systematically below 18% and 20%, respectively. Accuracy ranged from 106% to 115% for BPA-G, from 103% to 111% for BPS-G and from 93% to 105% for BPF-G with intra- and inter-day CV precision lower than 20%, indicating a good repeatability of our method (Table 3).

3.4.4. Stability and Carry-over

Stability during the residence time in the autosampler at 15 °C was assessed by assaying the labelled BP-Gs in low and high QC samples at t0, 2, 4, 6, 10, 12 and 24 h after extraction and dansylation. The resulting high QC concentrations were close to their nominal values for all the three BP-Gs at least 24 h. At low concentrations, Dn-BPS-G were stable over 24 h whereas Dn-BPA-G concentrations tended to decline at 24 h ($P = 0.07$) and Dn-BPF-G was significantly different from its initial value (t0) ($P < 0.05$, Table 3). These results underlines the need to assess the autosampler stability at two concentration levels. Indeed, the extracted matrix evolved over time and the low concentrations inexorably depended on these changes. By cautious, samples were assayed within 10 h after dansylation. Carry-over was assessed by injecting three solvent samples after a high QC concentration level (3.75 ng/mL). No carry over was observed in the first solvent sample.

3.5. Application of the method to the determination of fetal exposure

The suitability of the method was assessed by applying it to 44 umbilical cord samples from a French cohort. BPF-G was not detected in any of the samples. These cord plasma results are consistent with those of Pan et al 2020, who did not detect any BPF in 60 cord plasma samples (Table 4). However BPA-G and BPS-G were frequently detected in cord plasma (41% and 45%) with median values of 0.022 ng/mL (0.05 nM) and 0.101 ng/mL (0.23 nM), respectively (Table 4). The results recently obtained in birth cohort studies confirm the predominance of the conjugated forms of BPA-G and BPS-G in fetal plasma. Indeed, the concentration levels of total BPA measured after enzymatic hydrolysis in cord plasma were of the same order of magnitude as our BPA-G values: 0.051 ng/mL (0.22 nM) (Minatoya et al ., 2017) and 0.057 ng/mL (0.25 nM, Yamamoto et al., 2016), whereas unconjugated BPA was not detected or represented<10% of total BPA, respectively. Likewise, the total BPS concentrations measured in recent birth cohorts varied between 0.03 and 0.12 ng/mL (0.12–0.48 nM, Liu et al., 2017a, 2017b) with a geometric mean of 0.030 ng/mL (0.12 nM, Zhang et al. 2020) whereas no native BPS was detected in cord serum samples (Liu et al., 2017a).

4. Conclusion

The present study describes a novel method for simultaneous quantification of the glucurono-conjugated metabolites of BPA and its major substitutes, BPS and BPF, in plasma. To the best of our knowledge, this is

Table 4
Concentrations of BPA-G, BPF-G and BPS-G in cord plasma.

	Cord plasma (n = 44)		
	BPA-G	**BPS-G**	**BPF-G**
Nb values higher than LOD	18 (41%)	20 (45%)	0
Nb values higher than LOQ	7 (16%)	17 (39%)	0
Concentration range ng/mL	nd-0.089	nd-0.586	nd

nd: not detected.

the first time that dansyl chloride has been applied to bisphenol-glucuronides to increase the method sensitivity required for their direct quantification in human plasma. The experimental designs revealed optimal derivatization conditions that were milder than the conditions usually adopted for native bisphenols quantification. The proposed derivatization procedure associated with anionic exchange SPE considerably improved the sensitivity and selectivity of the method. The low detection limit allowed BPA-G and BPS-G concentrations to be determined in almost half of the 44 cord plasma samples, indicating widespread gestational exposure both to BPA and BPS. Maternal exposure at different stages of pregnancy now needs to be studied in order to understand the factors determining fetal exposure.

CRediT authorship contribution statement

C.A. Gély: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **A. Huesca:** Investigation, Validation, Writing - review & editing. **N. Picard-Hagen:** Investigation, Writing - review & editing. **P.L. Toutain:** Writing - review & editing. **A. Berrebi:** Investigation, Writing - review & editing. **G. Gauderat:** Investigation, Writing - review & editing. **V. Gayrard:** Investigation, Writing - original draft, Funding acquisition, Writing - review & editing. **M.Z. Lacroix:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank all the staff at the Gynecology-Obstetric Unit of Paule de Viguier Hospital, Toulouse, France. The authors would like to thank Sylvie Puel for her technical support.

Funding Source

This work was supported by the French Region Midi-Pyrénées (31000642), the French National Research Agency (ANR-13-CESA0007).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106429.

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