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Simultaneous quantification of bisphenol A and its glucuronide metabolite (BPA-G) in plasma and urine: Applicability to toxicokinetic investigations

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ABSTRACT

Bisphenol A (BPA) is a widely used plasticizer that can contaminate food and the wider environment and lead to human exposure. In humans, it is mainly metabolized to bisphenol A-glucuronide (BPA-G) and eliminated in the urine. As BPA causes adverse physiological effects at low doses, it is necessary to document the toxicokinetics of both molecules for risk assessment. Because BPA-G is not available as an analytical standard, it is usually quantified after the assay of BPA, following an enzymatic hydrolysis with β-glucuronidase. With this approach, two separate assays are required for BPA and BPA-G quantification, which can lead to critical pitfalls in terms of accuracy and analysis time. To overcome this problem, we have developed a new method for the isolation and purification of BPA-G from urine by flash chromatography. Large amounts of BPA-G (1 g) were isolated and characterized by mass spectrometry and NMR. This BPA-G is suitable for an use as analytical standard and enabled us to develop a novel method for the simultaneous quantification of BPA and BPA-G in biological matrices by UPLC/MS/MS. It has also been used for *in vivo* toxicokinetic studies in sheep. The method of quantification was validated according FDA guidelines and used to monitor the time course of plasma and urine concentrations of BPA or BPA-G following their administration. The simultaneous quantification of BPA and BPA-G was compared to the commonly used method for urine and plasma samples. For plasma samples, the results obtained with the direct assay of BPA-G were similar to those obtained by quantification after enzymatic hydrolysis. With urine samples, the simultaneous quantification appeared to be more suitable than the hydrolysis method for the BPA-G determination.

1. Introduction

Bisphenol A (BPA) is a monomer widely used in the production of epoxy resins and polycarbonate plastics used in food packaging. The human exposure to BPA occurs mainly by ingestion of food and water contaminated by residual monomers of BPA released from by food contact with the polymeric packaging materials. It has been shown that BPA can cause adverse effects in laboratory animals exposed to lower doses than the recommended tolerable dose intake (TDI 50 μg/kg/d) [1]. The polemic concerning the TDI established by the regulatory authorities and the scientific community might arise from the physiological differences between rodents and humans in terms of the toxicokinetics (TK) [2].

In humans, BPA is metabolized in the liver to its glucurono-conjugated form (BPA-G) and eliminated mainly in urine [3,4]. Glucuronidation is considered to be a detoxification mechanism, because BPA-G does not possess estrogenic activity [5]. The estimation of the transformation of BPA into BPA-G is of great concern for the risk analysis for human health based on animal studies [2], underlying the urgent need for rapid and reliable methods to assay BPA-G in different biological matrices. As a BPA-G analytical standard is not commercially available, BPA-G is usually quantified by LC/MS/MS by analysing the BPA produced after the hydrolysis of the BPA-G with commercial enzyme from *Helix Pomatia* which has both β-glucuronidase and aryl-sulfatase activities [6,7]. Therefore BPA-G concentrations are calculated by subtraction of the unconjugated BPA assayed before hydrolysis from the total BPA found after hydrolysis. Consequently two separated analyses of BPA are required to calculate the concentration of the BPA metabolite. Furthermore, this method has limitations linked to the fact that BPA-G is not discriminated from the other conjugated metabolites which can also be hydrolysed by β-glucuronidase (Sulfo-, glucosulfo-derivatives) [8]. Some laboratories have developed methods to

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isolate BPA-G from urine [3], bile [9] or liver microsomes [10]. These methods are time consuming and expensive and produce only limited amounts of BPA-G at any one time [3,9]. Toxicokinetic investigations involving the *in vivo* administration of BPA-G and its determination in several matrices and tissues often require large amounts of this compound. It is noteworthy that several instances including US NIEHS have been working on BPA metabolites synthesis, which raises the hope that other standards might be soon available for the scientific community to work with.

The first goal of the present study was therefore to develop a method to isolate and purify large amounts of BPA-G. During the last decade, hybrid systems of gravity column chromatography and conventional HPLC for rapid preparative chromatography with pre-packed cartridges have been commercialized as "Flash Chromatography" systems and cartridges. These systems allow higher sample loads than preparative HPLC with good separation of compounds from matrices and other contaminants. They were developed for the purification of synthetic products [11] but few studies have addressed the isolation of compounds from biological matrices [12]. Thus, in this study we applied the flash chromatography technique to purify BPA-G from urine after the administration of BPA to sheep.

The second goal of this study was to develop and validate an UPLC/MS/MS method according to the Food and Drug Administration (FDA) guidelines for the simultaneous quantification of BPA and BPA-G in urine and plasma. The method was evaluated for its suitability to monitor simultaneously BPA and BPA-G in biological matrices for TK investigations. Finally, this new method was compared to the common method using enzymatic hydrolysis for the determination of BPA and BPA-G.

2. Materials and methods

2.1. Chemicals

Bisphenol A, acetonitrile (AcN), methanol (MeOH), ethanol (EtOH), corn oil, Tris–HCl buffer, sodium chloride (NaCl), β-glucuronidase from *Helix Pomatia* and deuterated (d16) bisphenol A (BPA-d16) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Water was obtained from an ultrapure water (18.2 MΩ) system (Elga Labwater Veolia, Anthony, France).

Working concentrated solutions were prepared by serial dilutions of BPA and BPA-G in AcN/H_2O (50/50, v/v) to obtain final concentrations of each analyte of 10,000, 1000, 100, 10, 1, and 0.1 μg/mL.

Standard and quality control (QC) solutions were obtained by diluting working solutions in drug-free sheep plasma or urine to obtain concentrations ranging from 1 ng/mL to 500 μg/mL for the standard curve calibrators and from 2.5 ng/mL to 250 μg/mL for the QC samples. Calibrators and QC solutions were stored at −20 °C in 100 μL aliquots.

The stock solution of the internal standard (IS, BPA-d16) was prepared in AcN at a concentration of 1 mg/mL and stored at −20 °C. A new working IS solution was prepared each week by diluting the stock solution in ultrapure water and storing at 4 °C.

2.2. Animal treatment and sample collection

All animal procedures were carried out in accordance with accepted standards of humane animal care under agreement number 31-242 for animal experimentation from the French Ministry of Agriculture.

2.2.1. Oral administration of BPA for urine collection and sample processing

For oral administration, BPA powder was extemporaneously dissolved in EtOH/corn oil (1:9, vol:vol) at 100 mg/mL. A BPA dose of 100 mg/kg body weight (b.w.) was orally administered to one ewe (61 kg) using a naso-oesophagian tube. Total urine samples were collected 1, 2.5 and 3.5 h after the oral administration via a Foley catheter placed in the urethra and immediately chilled in ice. A volume of approximately 250 mL was obtained for BPA-G isolation with flash Chromatography.

2.2.2. Intravenous administration of BPA and BPA-G for pharmacokinetic studies and sample processing

BPA powder was extemporaneously dissolved in EtOH/propylene glycol (1:49, vol:vol) at 5 mg/mL and purified BPA-G solution (40 mg/mL) was diluted in EtOH/NaCl buffer 0.1% (1:49, vol:vol) to give 3 mg/mL. A BPA dose of 0.5 mg/kg b.w. was intravenously (IV) administered to three ewes (61.5 ± 4.0 kg) via an indwelling catheter inserted into the left jugular vein just prior to the BPA administration. In a second trial, BPA-G was infused intravenously for 24 h at a constant rate of 0.68 μg/(kg min) corresponding to a total BPA-G dose of 1 mg/(kg d) to two ewes (35 and 40 kg) into the left jugular vein via an indwelling catheter as previously described [13].

Serial blood samples were collected before and at 2, 4, 8, 15, 20, 30 min and 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 36 and 48 h after BPA administration and every 30 min during the second trial of BPA-G infusion starting 21 h after the beginning of the infusion and 2, 4, 8, 15, 30, 45 min, 1, 1.5, 2, 4, 6, 8, 10, 24, 32 and 48 h after the end of the infusion.

Blood samples were collected in heparinized tubes by direct puncture of the right jugular vein, immediately chilled in ice and centrifuged for 30 min at 3000 × *g* at 4 °C. The resulting plasma was stored at −20 °C until assay.

After the intravenous administration of BPA, urine samples were collected as described above at 1, 2, 3, 6, 9, 12, 16, 20, 24, 28, 32 and 36 h.

All urine samples were centrifuged for 10 min at 3000 × *g* at 4 °C and then aliquoted into glass tubes in 1 mL fractions and stored at −20 °C.

2.3. BPA-G purification by flash chromatography

Pooled urine samples were concentrated with a rotavapor at 50 °C to obtain a volume of approximately 30 mL. Pre-concentrated urine samples were injected on the flash chromatographic system with a H_2O/MeOH gradient elution (Supplementary material). The collected BPA-G fractions (10 mL) were pooled and dried under a stream of air at 50 °C. The dried residue was dissolved in 4 mL of EtOH, filtered under vacuum, and stored at −20 °C.

2.4. Analytical instrumentation and conditions

2.4.1. Instrumentation

BPA and BPA-G identification and quantification were performed with an Acquity ultra performance liquid chromatography (UPLC®) coupled to a Xevo® triple quadrupole mass spectrometer and an Acquity PDA detector (Waters, Milford, MA, USA). BPA, BPA-G and the internal standard were separated on an Acquity BEH® C18 column (2.1 mm × 100 mm; 1.7 μm; Waters). Samples were ionised in negative electrospray ionisation mode (ESI^-). The capillary voltage and source temperature was set at 2.2 kV and 150 °C, respectively. The desolvatation temperature and nitrogen flow rate were set at 650 °C and 1200 L/h, respectively. Argon was used as the collision gas at a flow rate of 0.12 mL/min.

2.4.2. BPA-G identification

The BPA-G fractions collected by flash chromatography were eluted on the C18 column with a H_2O/AcN gradient ($t(0 \rightarrow 1$ min): 10% AcN; $t(1 \rightarrow 10$ min): 90% AcN). BPA-G was identified by diode

array detection (DAD) with wavelengths ranging from 190 to 500 nm; MS full scan with a mass range of 50–800 Da. The MS/MS daughter scan was set at $m/z = 403$ corresponding to the $[M-H]^-$ BPA-G ion parent with a collision energy of 20 eV and with a mass range of 50–500 Da. BPA-G chemical structure was checked by ^1H and ^{13}C NMR (Supplementary material).

2.4.3. Methods of quantification

2.4.3.1. Quantification after enzymatic hydrolysis. The aliquots of purified BPA-G obtained from the first purification run were diluted a thousand fold in sheep plasma. Fifty microliters of these BPA-G and of BPA spiked plasma samples were incubated for 2 h at 37 °C with 30 μL of β-glucuronidase from *Helix Pomatia* and 420 μL of Tris–HCl buffer (50 mM, pH 7.4). After incubation, the samples were centrifuged at 20,000 × *g* at 4 °C for 10 min. BPA in supernatant (100 μL) was quantified as described below. Aliquots of the fractions obtained from the subsequent purification runs were directly quantified with spiked BPA-G plasma samples as described below.

2.4.3.2. Simultaneous BPA and BPA-G quantification. BPA and BPA-G in urine or plasma samples (100 μL) were purified by protein precipitation/dilution with 150 μL AcN and 50 μL of the IS (BPA-d16). BPA and BPA-G were eluted on the C18 column with a H_2O/AcN gradient ($t(0 \rightarrow 0.5$ min): 10% AcN; $t(0.5 \rightarrow 4$ min): 90% AcN). The quantification of BPA and BPA-G was by using Multiple Reaction Monitoring, MRM transitions and collision energies (Ecoll) for quantification were 227 > 212 Ecoll = 28 eV for BPA, 241 > 142 Ecoll = 20 eV for BPA-d16 and 403 > 227 Ecoll = 30 eV for BPA-G. Chromatographic data were monitored by Targetlynx® software (Waters, Milford, MA, USA).

2.5. Validation procedure

BPA and BPA-G standard curves and quality controls (QC) were prepared separately to avoid potential contamination. The quantification method for each molecule was validated according to the Food and Drug Administration (FDA) guidelines in term of selectivity, linearity, repeatability and reproducibility [14]. Selectivity was tested by comparing six blank plasma (or urine) chromatograms with chromatograms at the limits of quantification (LOQ). Limit of detection (LOD) was defined as the estimated concentration with a signal equal to three times the mean noise of six blank matrices at the retention time of the analytes. Calibration curves were calculated using WinNonlin® 5.2 sofware (Pharsight Corporation, Mountain View, CA, USA). Linear ($Y = aX + b$) and quadratic ($Y = aX^2 + bX + c$) models were tested with weightings: 1, $1/X$ and $1/X^2$ (X = nominal concentration). The linearity of the calibration curve was assessed using three approaches: (1) the calculation of the relative standard deviation between the nominal concentration and the concentration obtained with the model (RSD%), which should be lower than ±15% (except at the LOQ, ±20%), (2) the visual inspection of the residual distribution which should be randomized around the mean [15] and, (3) a lack of fit test to check the goodness of fit of the model [16,17]. The LOQ were defined as the lowest concentration of the calibration curve that could be quantified with a precision lower than 20% and within an accuracy range of 80–120%. Intra-day and inter-day precisions and accuracy of BPA and BPA-G were calculated from three different days and with six replicates of QC samples at three concentration levels (low, middle and high) covering the range of standard curve concentrations. Matrix effects were quantitatively estimated with the matrix factor (MF). MF is defined as the ratio of analyte peak area in the presence of matrix ions to the analyte peak area in the absence of matrix ions [18]. BPA-G matrix factor was calculated at three concentrations levels 0.01; 0.1 and 1 μg/mL for plasma and 1; 10 and 100 μg/mL for urine covering the range of each calibration curve.

3. Results and discussion

3.1. BPA-Gluc purification

3.1.1. Optimization of flash chromatography

Normal phase flash chromatography used with silica cartridges was first checked for its ability to separate BPA-G from BPA and other interfering urine components. Thin layer chromatography (TLC) was used for the mobile phase selection [12] and the best separation between BPA and BPA-G was obtained with a CH_2Cl_2/EtOH (70/30) mixture. Gradient elution with this selected mixture and sample loading were optimized according the general method used for the transposition of TLC to flash chromatography [19]. However, because of its high polarity, BPA-G was strongly retained on the silica and eluted in the last fractions of the gradient, which made accurate UV detection difficult. Consequently, reverse phase flash chromatography was tested with a C18 cartridge. The solvent and gradient elution developed for semi-preparative chromatography were adapted for flash chromatography [12]. A good separation between BPA, BPA-G and interfering urine components was obtained with a H_2O/MeOH gradient elution in less than 40 min and after injection of 4 mL of pre-concentrated urine (Supplementary material).

3.1.2. BPA-Gluc identification

The fractions collected by flash chromatography were pooled and analyzed by LC–UV and LC–MS/MS. For both methods of detection, no interference was observed on the chromatograms, with only one peak that was eluted at $t_R = 2.5$ min. With UV detection, this peak had maximum absorbance at the following wavelengths: $\lambda_{abs} = 190$, 230 and 280 nm corresponding to the transitions of the phenol function. In mass spectrometry, one peak was observed at $t_R = 2.5$ min at $m/z = 403$ which corresponded to the deprotonated parent ion $[M-H]^-$ of BPA-G. MS/MS was performed at $m/z = 403$ with 20 eV of collision energy; the resulting fragments gave characteristic ions at $m/z = 227$ and $m/z = 175$ corresponding to the BPA and glucuronic acid moieties, respectively and at $m/z = 113$ corresponding to the loss of CO_2 and H_2O from the glucuronic acid moiety (Fig. 1). Chemical structure was confirmed by ^1H and ^{13}C NMR and results were similar to those described in the literature [5,9] (Supplementary material).

3.1.3. Calculation of the amount of purified BPA-G

Initially the BPA-G obtained from the first purification was quantified by UPLC/MS/MS after enzymatic hydrolysis with β-glucuronidase using a BPA spiked plasma standard curve (1–100 μg/mL). The BPA concentration measured after enzymatic hydrolysis was 22.7 ± 1.0 mg/mL corresponding to 40.3 ± 1.8 mg/mL of BPA-G after correction for the difference in molecular weights. For this first attempt, the method of BPA-G purification appeared to be promising in terms of amount. Fractions were then directly quantified with a spiked BPA-G plasma standard curve (0.01–20 μg/mL), which showed that 1036 mg of BPA-G could be purified from one ewe. The time-course of the presence of BPA-G in the urine indicated that the yield could be optimized by collecting urines samples for much longer after BPA administration than 3.5 h (Fig. 4).

3.2. Simultaneous quantification of BPA and BPA-G

3.2.1. Optimization of the UPLC/MS/MS assays

The extraction procedure for the BPA and BPA-G was a simple AcN dilution/precipitation for plasma and urine samples to mini-

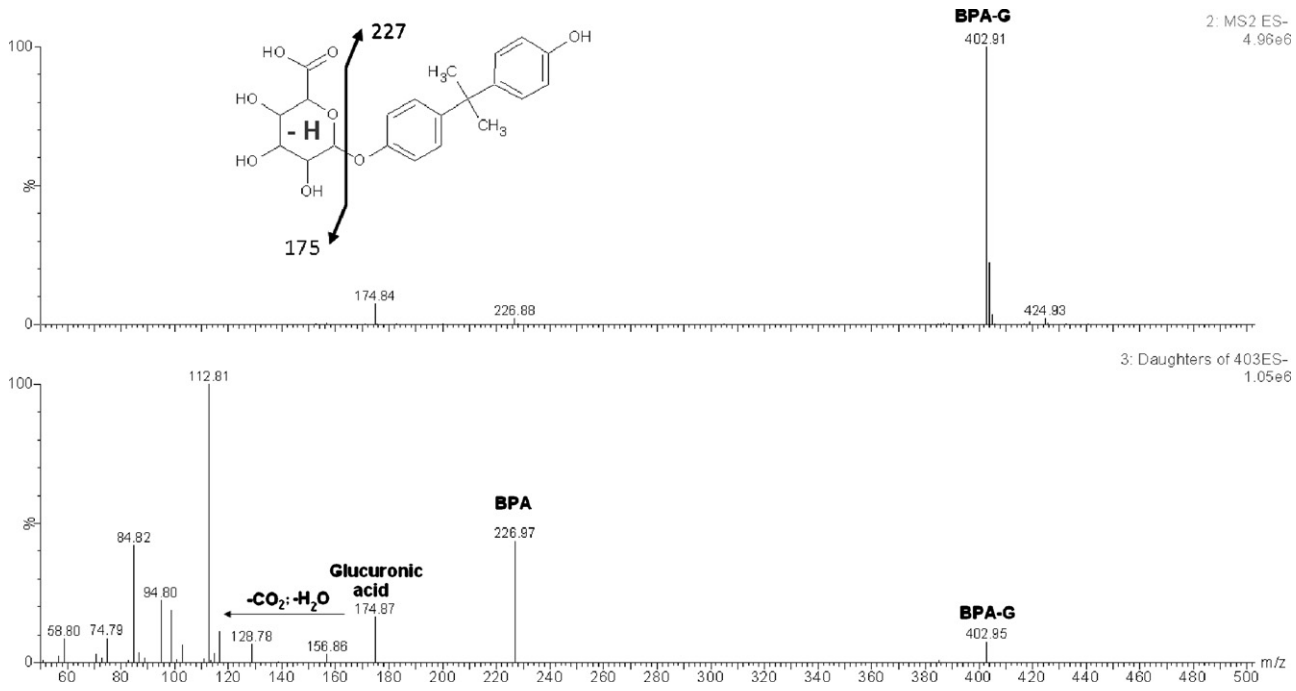

Fig. 1. MS and MS/MS spectra obtained for the collected BPA-G fractions after flash chromatography purification (MS/MS: $m/z = 403$; Ecoll = 20 eV).

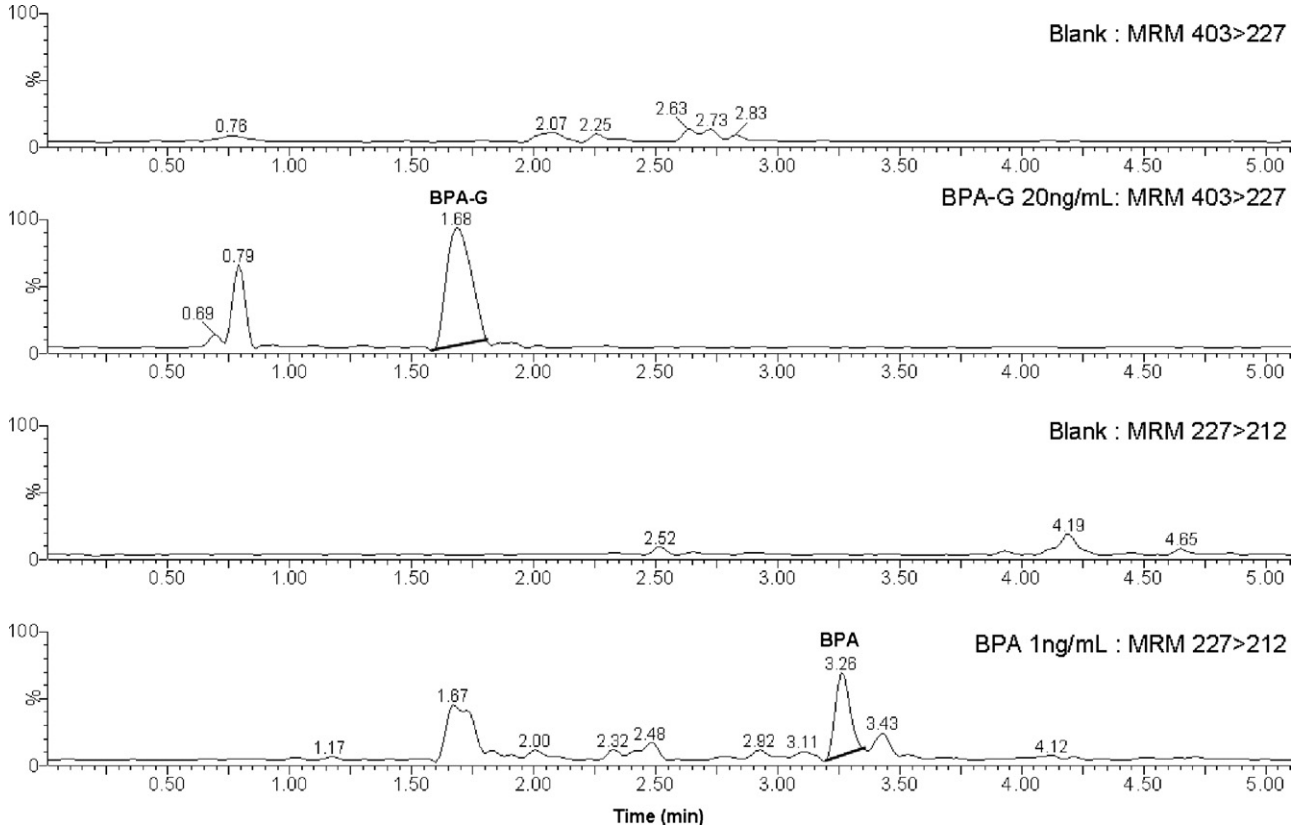

Fig. 2. MRM chromatograms of blank and spiked sheep plasma (1 ng/mL of BPA and 20 ng/mL of BPA-G). The retention times of BPA and BPA-G were 1.68 min and 3.26 min, respectively.

mize potential sources of contamination by BPA. No interference in blank matrices was detected at the transition of BPA and BPA-G with this extraction procedure (Fig. 2). The elution conditions were optimized to separate BPA from BPA-G and BPA-G from the solvent front in the shortest run time (less than 5 min). The retention times of BPA and BPA-G were 1.68 min and 3.26 min, respectively (Fig. 2).

3.2.2. Validation of the methods

The ranges of the calibration curve and validation results are summarized in Table 1. Partial validation was successful for both matrices (plasma, urine) of piglets, dogs, rats and mice (data not shown), attesting to the robustness of the method and its suitability for different species. For BPA quantification, the accuracy

Table 1
Validation results of BPA and BPA-G in sheep plasma and urines.

Matrices	BPA Nominal concentration (μg/mL)	Mean (μg/mL)	Accuracy	Precision RSD%		BPA-G Nominal concentration (μg/mL)	Mean (μg/mL)	Accuracy	Precision RSD%	
Calibration range				Intra-day	Inter-day				Intra-day	Inter-day
Plasma	LOD[a] (*S*/*N* = 3)	0.0006				LOD[a] (*S*/*N* = 3)	0.002			
	LOQ (*n* = 6)					LOQ (*n* = 6)				
BPA	0.001	0.00108	108%	12%		0.020	0.017	102%	6%	
0.001–1 μg/mL										
BPA-Gluc	QC (*n* = 18)					QC (*n* = 18)				
0.02–20 μg/mL										
	0.0025	0.0024	97%	12%	12%	0.050	0.051	103%	12%	12%
	0.025	0.024	95%	7%	11%	0.750	0.825	110%	8%	8%
	0.250	0.252	101%	6%	3%	7.50	8.11	108%	8%	8%
Urine	LOD[a] (*S*/*N* = 3)	0.002				LOD[a] (*S*/*N* = 3)	0.010			
	LOQ[b] (*n* = 6)					LOQ[b] (*n* = 6)				
BPA	0.025	0.027	109%	10%		1	0.97	97%	15%	
0.025–10 μg/mL										
BPA-Gluc	QC (*n* = 18)					QC (*n* = 18)				
1–500 μg/mL										
	0.075	0.079	105%	10%	10%	2.50	2.47	99%	17%	17%
	0.25	0.266	106%	9%	9%	25	23.9	96%	18%	18%
	2.50	2.54	101%	11%	11%	250	227	91%	15%	15%

[a] LOD estimated from 6 blanks and with signal to noise ratio *S*/*N* = 3.
[b] LOQ established in urine as the lowest calibration point necessary for toxicokinetic investigations.

ranged from 95% to 109% and the dispersion was lower than 12%. The accuracy range of BPA-G was 91–110% with precision lower than 15% for plasma and around 15–18% for urine samples.

The LOQ was set and validated for the lowest value of the calibration curve chosen for its relevance toward toxicokinetic investigation. For BPA-G in urine, this LOQ was 1000-fold higher than those reported in the literature after enzymatic hydrolysis [7,20]. We did not aim at determining the lowest concentration of BPA-G that could be measured with suitable precision and accuracy to fulfill FDA requirements [14]. LODs were 1 and 10 ng/mL in plasma and urine, respectively (Table 1) suggesting that it is possible to optimize the assay to obtain a LOQ within the ng/ml range.

Since the matrix effect observed for stable isotope-labeled internal standard is similar to those observed for the matching analyte [18], matrix factor was calculated in both matrices only for BPA-G. In plasma samples, BPA-G matrix effects were negligible (MF = 1.04 ± 0.14). In contrast, BPA-G MF in urine samples was 0.57 ± 0.09 suggesting BPA-G ionization suppression. As for BPA, this matrix effect could be overcome by the use of internal standard of BPA-G labeled with a stable isotope. Purification of such an internal standard with our method has been envisaged but it would require large amounts of isotope-labeled BPA as BPA-G is extracted from sheep urine. It is noteworthy that this matrix effect appeared to be similar for three levels of BPA-G concentrations covering the calibration range limiting its potential impact on assay performance. As mentioned above and based on the LOD evaluation, our method could be improved to be suitable for BPA and BPA-G residue screening in human. Nevertheless, this method gave sensitivity and reproducibility suitable for TK investigations.

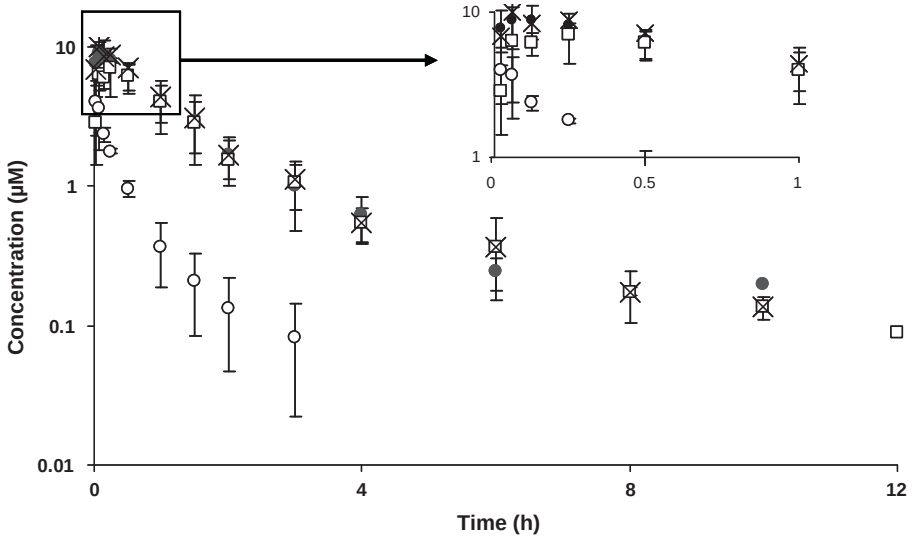

Fig. 3. Mean + SD plasma molar concentrations versus time of BPA (○), BPA-G (□) and BPA-G + BPA (x) after direct measurement, and BPA (●) obtained after an enzymatic hydrolysis, after an IV bolus administration of BPA (0.5 mg/kg (b.w.)) to sheep (*n* = 3).

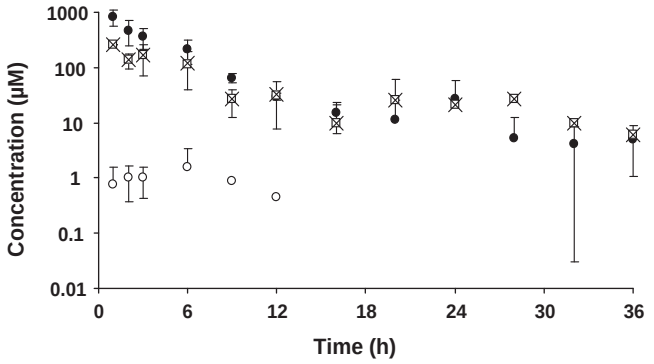

Fig. 4. Mean + SD urine molar concentrations of BPA (○), BPA-G (□) and BPA-G + BPA (x) after direct measurement and BPA (●) obtained after an enzymatic hydrolysis, versus time after an IV bolus administration of BPA (0.5 mg/kg (b.w.)) to sheep ($n = 3$).

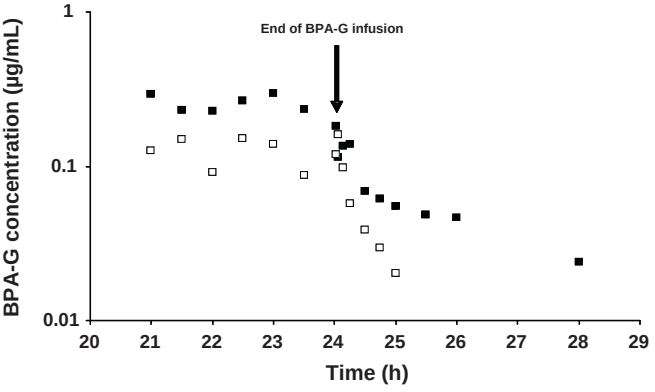

Fig. 5. Plasma concentrations of BPA-G versus time after an IV infusion of BPA-G (1 mg/(kg.d) (b.w.)) to sheep ($n = 2$).

3.3. Applicability of the method for toxicokinetic studies of BPA and BPA-G

Two experiments were carried out to verify the suitability of the direct measurement of BPA-G for TK studies.

Firstly, as shown in Figs. 3 and 4, BPA and BPA-G were quantified up to 12 h in plasma and up to 36 h in urine after an IV bolus administration of BPA (0.5 mg/kg b.w.) in sheep. Our method appeared to be suitable to determine the time course of BPA and BPA-G concentrations in both urine and plasma in the same run without the use of enzymatic hydrolysis.

Secondly, BPA and BPA-G were assayed after an IV infusion of BPA-G (1 mg/(kg d)). The mean steady-state BPA-G concentration (C_{SS}[BPA-G]) was 0.185 ± 0.072 µg/mL. The direct BPA-G quantification enabled the time-course of BPA-G concentrations to be monitored after the end of the infusion. BPA-G concentrations decreased rapidly and were below the limit of quantification (0.02 µg/mL) 4 h after the end of the infusion (Fig. 5). Interestingly, no BPA was detected, suggesting that BPA-G was neither significantly hydrolysed into BPA *in vivo*, nor during the sample extraction procedure.

3.4. Comparison between β-hydrolysis of BPA-G and direct BPA-G quantification

BPA-G concentrations in plasma samples after BPA-G infusion ($n = 29$) were measured after enzymatic hydrolysis using either a hydrolysis BPA-G standard curve or enzyme-treated BPA curves. As shown in Fig. 6, the values obtained with the two calculations were perfectly correlated according to a linear curve equation with an R^2 of 0.9999 and a slope of 0.493, close to the ratio of the molecular weights of BPA to BPA-G (0.564). Thus the difference between the two slopes accounted for less than 7% of the expected value.

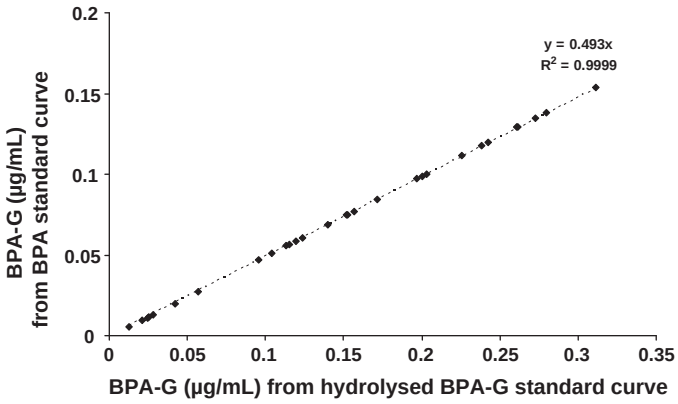

Fig. 6. Sheep BPA plasma concentrations ($n = 29$) after BPA-G IV infusion (1 mg/(kg.d) (b.w.)) measured by using a hydrolysis BPA-G standard curve (*X*-axis) versus the same BPA-G plasma samples measured with an enzyme-treated BPA curve (*Y*-axis).

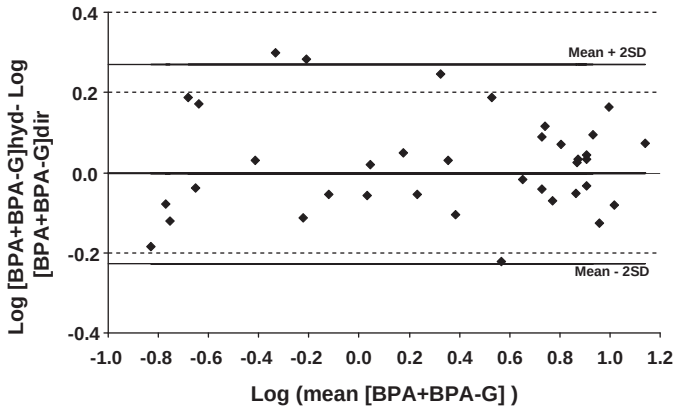

Fig. 7. Log-transformed Bland–Altman plots of BPA + BPA-G between the direct measurement method (dir) and the enzymatic hydrolysis method (hyd) for the corresponding BPA + BPA-G plasma concentration. The *X*-axis is the logarithmic mean of the BPA + BPA-G measured with the two methods and the *Y*-axis is the log-transformed difference between the two methods.

BPA-G and BPA concentrations in sheep plasma and urines following an IV bolus of BPA were evaluated in the same samples with both the enzymatic hydrolysis method and the simultaneous direct measurement method (Figs. 7 and 8). Log-transformed Bland–Altman plots showed that the residuals were randomly distributed around the mean value of the two measurements for plasma samples (Fig. 7) attesting that the two methods were in good agreement [21]. Moreover, this good correlation between the two methods clearly suggests that the contribution of BPA-sulfate to total BPA concentration if any is negligible.

However in urine, the agreement between the two methods was biased particularly for the highest values (Fig. 8). Indeed, the values obtained after hydrolysis were systematically higher than those obtained by direct measurement. It can be assumed that metabolites other than BPA-G can be eliminated in urines [8] and that these metabolites are also sensitive to hydrolysis by β-glucuronidase. The contribution of these BPA metabolites formed by hydrolysis might be sufficient in high concentrations to lead to an overestimation of BPA-G.

Furthermore, Table 2 shows that when measuring the total amounts of BPA found in urines over 36 h following the IV bolus administration of BPA as a percentage of the administered

Table 2
Percentage of BPA dose found in urine with the two methods.

Sheep	% BPA dose			
	BPA	BPA-G	BPA hydrolyzed	Other Metabolites
1	<0.01%	24.7%	41.1%	16.4%
2	<0.01%	25.4%	82.0%	56.6%
3	<0.01%	18.2%	34.1%	15.9%
CV%		17%	49%	79%

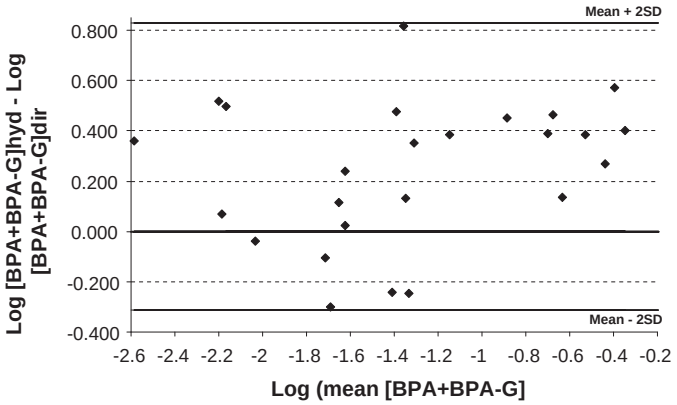

Fig. 8. Log-transformed Bland–Altman plots of BPA + BPA-G between the direct measurement method and the enzymatic hydrolysis method for the corresponding BPA + BPA-G urine concentration.

dose, the hydrolysis method led to an inter-individual coefficient of variation much higher than the direct method. This could be explained by modulation of the efficiency of the enzymatic hydrolysis by different factors such as contamination or infections [22,23]. From this standpoint, the direct measurement method can be considered as more reliable than the enzymatic hydrolysis one.

4. Conclusion

The present study describes a novel method of BPA-G purification from urine and a new UPLC/MS/MS method for the simultaneous quantification of BPA and BPA-G in urine and plasma. This new approach of purification by flash chromatography enabled the rapid purification of large amounts of BPA-G in the gram range with a purity compatible with standard grade norms [24]. The simultaneous quantification of BPA and BPA-G by UPLC/MS/MS has been validated according the criteria of the FDA guidelines. The particular advantage of this method is its ability to quantify both BPA and BPA-G rapidly, in less than 5 min and in a single run. This is a real benefit in terms of the time devoted to sample preparation and analysis. This method is all the more useful in that (1) it allows BPA-G to be specifically assayed independently of any other conjugated BPA metabolites and (2) it avoids the problems of factors likely to alter β-glucuronidase activity and thereby the accurate measurement of BPA-G in complex matrices such as urine or tissue extracts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.07.040.

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