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Factors Affecting the Accuracy of Bisphenol A and Bisphenol A-Monoglucuronide Estimates in Mammalian Tissues and Urine Samples

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ABSTRACT Bisphenol A (BPA) (CAS Number 80–05-7; EINECS Number 201–245-8) is used in the production of plastics having food contact applications. Some biomonitoring studies have reported free BPA in blood or urine of humans. Since complete first-pass metabolism of orally administered BPA to BPA-monoglucuronide (BPA-G) occurs in humans, the presence of free BPA in human specimens raises questions as to the origin and/or possible sources of the free BPA. We hypothesized that BPA-G instability during specimen collection and analysis contributes to the presence of free BPA in the biological samples. Investigation of the in vitro hydrolysis of BPA-G in blood plasma, tissue homogenates, and diluted urine from laboratory rats and in aqueous/organic solutions commonly used for extraction in BPA analyses lent support to the hypothesis of BPA-G instability as a possible source of free BPA determinations in the biological specimens. Hydrolysis of BPA-G occurred at neutral pH and room temperature in diluted urine and in rat placental or fetal tissue homogenates at room temperature. Hydrolysis of BPA-G in aqueous/organic solutions began within minutes at pH 2 and 80°C. BPA-G was degraded to an unidentified compound in a urine/water mixture or when stored in a 25/75 mixture of urine/acetonitrile at pH 9 at either 22 or 80°C. Based upon these experiments, it was concluded that methods demonstrating BPA-G stability or accounting for its instability during analysis are warranted in studies designed to measure free BPA in biological specimens.

KEYWORDS Bisphenol A; Bisphenol A Glucuronide; Excreta; Mammalian Tissues

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INTRODUCTION

The major metabolite of BPA produced by both rats and mice was identified as BPA-G (Pottenger et al. 2000; Upmeier et al. 2000; Snyder et al. 2000; Zalko et al. 2003). A carefully conducted pharmacokinetic study in human volunteers has shown that oral administration of a 5-mg dose of d_{16}-BPA resulted in complete first-pass metabolism to BPA-G; free BPA was not detected in the

blood at any time postdosing and the subsequent urinary excretion of BPA-G was rapid and complete ($t_{1/2} = 5.4$ h) (Völkel et al. 2002). Following the study of Völkel et al. (2002), Tsukioka et al. (2004) administered lower oral doses of either 50 or 100 μg of ^{16}d-BPA to human volunteers and reported that virtually 100% was excreted as BPA-G in the urine within 24 h postdosing. Völkel et al. (2005) conducted an additional in vivo pharmacokinetic study in six human subjects (three men and three women) using an orally administered dose of 25 μg BPA/person. In male subjects, 93 $\pm$ 19 nmol (85% of the dose) and in female subjects 83 $\pm$ 16 nmol (75% of the dose) were recovered in the urine as BPA-G with in 5 h postdosing. An elimination half-life of 4 h was calculated from the excretion rates. Free BPA was found in two of the urine specimens collected (one man at 1 h and one woman at 5 h) at concentrations less than 1 ng/mL; amounts equivalent to less than 2% of the administered dose. The data from these three studies were generally consistent, indicating that there is rapid and virtually complete presystemic elimination of BPA in humans by first-pass metabolism to BPA-G.

In individuals of normal health status and without direct, known exposure to BPA, some investigators have reported trace amounts (mean or median values of ~1 to 2 ppb or less) of unmetabolized BPA in serum (Sajiki et al. 1999; Inoue et al. 2000; Okhuma et al. 2002; Shi et al. 2004), maternal plasma, placenta and cord blood (Schoenfelder et al. 2002), umbilical cords (Takada et al. 1999), maternal serum and amniotic fluid (Yamada et al. 2002), and urine (Ouchi and Watanabe 2002; Matsumoto et al. 2003; Kim et al. 2003; Tsukioka et al. 2004; Ye et al. 2005). Some of these studies have also reported that unmetabolized BPA was not detectable in normal human serum in some or all individuals sampled (Ohkuma et al. 2002; Shi et al. 2004) or that only conjugated metabolites of BPA (BPA-G and/or BPA-SO_4) were present in human urine (Brock et al. 2001; Ouchi and Watanabe 2002). BPA was not detected in human fat from 21 subjects or in the endometrium of 22 out of 23 individuals (Shaefer et al. 2000).

It has been previously shown that glucuronide conjugates of xenobiotics can be hydrolyzed under certain conditions of sample preparation and analysis to release the aglycone (Bartels et al. 1997). We hypothesized that hydrolysis of BPA-G during the sample collection and/or the preparation and analysis of BPA in mammalian tissues or plasma might have occurred in at least some, if not all, of the studies cited above. In order to test our hypothesis, we investigated the stability of BPA-G in aqueous/organic solutions at two different pH conditions and at two temperatures. The conditions were chosen to reflect the range of sample preparation conditions previously reported in the scientific literature for the analysis of BPA in mammalian tissues or urine. In addition, the stability of BPA-G in blood plasma and homogenates of rat placenta and fetal tissue was determined following its direct addition to plasma or tissue homogenates from rats not dosed with BPA. For comparison, the stability of BPA-G found in the placenta homogenates following a single oral dose of BPA to pregnant rats was also determined.

MATERIALS AND METHODS

Test Materials and Solvents

Radiolabeled ^{14}C-BPA-G was obtained as a urinary metabolite from two female Fischer 344 rats administered ^{14}C-BPA by gavage at a dose of 100 mg/kg body weight. Immediately postdosing, the rats were housed individually in glass, Roth-style metabolism cages and the urine voided over the first 24 h postdosing was frozen immediately in a vessel chilled on dry ice. The urine obtained from both rats was pooled to produce a "stock solution" containing both ^{14}C-BPA-G and ^{14}C-BPA. The stock solution was determined by liquid scintillation counting (LSC) to contain 2921 dpm/μL urine (5.4 ng-eq BPA/μL). Analysis by high-performance liquid chromatography with radioactivity monitor (HPLC/RAM) found that the radioactivity in the pooled urine was about 97% ^{14}C-BPA-G and about 3% ^{14}C-BPA as observed in previous studies (Pottenger et al. 2000). The ^{14}C-BPA-G was found to be stable when stored in rat urine under neutral pH conditions at –80°C for a minimum of 42 days.

Two lots of uniformly ring-radiolabeled ^{14}C-BPA were obtained from Moravek Biochemical, Brea, California. The first (Lot 129–032-056) was used to dose the female Fischer rats to obtain BPA-G as a urinary metabolite and had a specific activity of 56 mCi/mmole. A second synthesis of ^{14}C-BPA (Lot 223A-261–200), specific activity 200 mCi/mmole, was used to dose female Sprague-Dawley rats for the study of BPA-G stability when produced and distributed to tissues in vivo. Both lots had a radiochemical purity of

greater than or equal to 98.8% as determined by HPLC analysis prior to use.

All solvents used were obtained from Fisher Scientific, Pittsburgh, Pennsylvania, and were of HPLC grade. Water was deionized and purified using a Milli-Q Water Purification System (Millipore Corporation, New Bedford, Massachusetts).

Animals, Husbandry, and Euthanasia

Female Fischer 344 rats (12 weeks of age, weighing 140 to 156 g at the time of dosing) were obtained from Charles River Laboratories, Raleigh, North Carolina. Time-mated Sprague-Dawley (SD) female rats (used on gestation day [gd] 16) were obtained from Hilltop Laboratories, Scottsdale, Pennsylvania. Following dosing, pregnant rats were housed individually in tubs with corncob bedding, 40 to 70% relative humidity, 21 to 23°C, until sacrifice at 15 min postdosing. All animals (dosed and controls) were humanely euthanized via asphyxiation with CO_2, followed by cardiac puncture. Following removal by cesarean section, rat fetuses were humanely euthanized by the sublingual deposition of Socumb-6 GR sodium pentobarbital solution (The Butler Company, Columbus, Ohio).

BPA-Glucuronide Stability at Various Physicochemical Conditions

The solution used for the study of BPA-G stability at four different physicochemical conditions was prepared by mixing 2 mL of the stock solution containing the radiolabeled test materials and 6 mL of acetonitrile. The urine was denatured with acetonitrile to minimize the potential for enzymatic or other hydrolysis. The denatured solution was filtered with a Whatman 0.2 μm syringe filter and stored in a sealed glass container. Based on the dpm/μL in the stock solution, the concentration of radioactivity as ^{14}C-BPA-G/^{14}C-BPA in the urine/acetonitrile solution was calculated to be 730 dpm/μL (1.339 ng-eq BPA/μL).

Duplicate samples, were prepared for analysis at 1, 4, 8, and 24 h. Samples were held at either room temperature (approximately 22°C) or 80°C with a pH of either 2 or 9 to determine the stability of BPA-G under these four different physicochemical conditions. Each sample consisted of a 200-μL aliquot of the urine/acetonitrile solution transferred to a 1.5-mL glass vial. Adjustment of the pH was done with 10 μL of concentrated HCl (pH 2) or 10 μL of concentrated ammonium hydroxide (pH 9). The pH was confirmed using Colorphast® pH indicator strips (EMD Chemicals, Gibbstown, New Jersey). The elevated temperature samples were placed into a heating block held at 80°C for the specified times. Samples specified as 22°C were held at room temperature in a laboratory with the temperature thermostatically controlled and monitored by LabVIEW (Version 1.02, National Instruments Corporation, Austin, Texas). The average temperature of this laboratory was 21.9°C ± 0.5°C for 8640 data points over 30 days. A thermal "control" (TC) consisting of a 200-μL aliquot of the urine/acetonitrile solution without pH adjustment, that is, neutral pH, was prepared for each temperature and analyzed after 24 h to determine the effect of temperature alone. These samples and thermal "controls" were injected directly onto the HPLC for analysis as described below.

Stability of BPA-Glucuronide Added to Biological Matrices Obtained from Control Animals

Controls

The controls for the study of BPA-G stability in biological matrices were samples of the stock solution containing both ^{14}C-BPA-G and ^{14}C-BPA mixed with deionized water (1:5), held at neutral pH and 25°C.

Placenta

Samples for the study of BPA-G stability in the placenta were prepared using pooled placental tissue homogenates obtained from control (not dosed with BPA) pregnant female SD rats. The pregnant dams were sacrificed on gd 16 (placentas were pooled from each animal at sacrifice but not pooled across dams). The placenta homogenates consisted of one part tissue and two parts water. The tissue and water were cooled on ice during homogenization (approximately 1 min) using a Sonifier Cell Disrupter, model W140 (Misonix Incorporated, Farmingdale, New York) and then immediately frozen and stored at −80°C until used.

Each sample consisted of a 500-μL aliquot of tissue homogenate transferred to a 4-mL glass vial and fortified with 100 μL of stock solution containing 2921 dpm/μL of ^{14}C- BPA/^{14}C- BPA-G as described above. This produced a sample concentration of 89 ng-eq BPA/mL. The vials were sealed with a Teflon-lined cap and held at room temperature until extraction

and analysis. Samples were prepared for extraction and analysis at 0, 1, 3, 5, and 24 h as described below. Thermal control samples, consisting of 500 μL of water fortified with the same amount of stock solution as the samples of placenta homogenates, were also prepared for extraction and analysis at 0 and 24 h.

Fetal Tissue

Samples for the determination of BPA-G stability in fetuses were prepared in pooled fetal tissue homogenates prepared from control (not dosed with BPA) pregnant female SD rats sacrificed on gd 16. Fetuses were pooled from each dam during collection and not pooled across dams. The tissue homogenate consisted of one part tissue and one part water by weight and was homogenized as described above, then immediately frozen and stored at −80°C until used. Samples were prepared as described above for placenta homogenates and were extracted and analyzed at 0, 1, 3, 5, and 24 h. Thermal control samples, consisting of 500 μL of water fortified with the same amount of stock solution as the samples, were also prepared for extraction and analysis at 0, 1, 3, 5, and 24 h.

Plasma

Samples for the determination of BPA-G stability in plasma were prepared in plasma obtained from control (not dosed with BPA) pregnant female SD rats sacrificed on gd 16. Blood was obtained from these rats via cardiac puncture following asphyxiation induced with CO_2. The blood was collected in vacutainers containing 143 USP units of heparin as an anticoagulant and centrifuged at 1200 g for 5 min to obtain blood plasma. The plasma obtained from each animal was then pooled for use in this study. Each sample consisted of a 500-μL aliquot of the pooled plasma transferred to a 4-mL glass vial fortified with 100 μL of the stock solution containing 2921 dpm/μL ^{14}C-BPA/ ^{14}C-BPA-G. This produced a sample concentration of 89 ng-eq BPA/mL. The vials were sealed with Teflon-lined caps and held at room temperature until extraction and analysis as described below. Samples were prepared for extraction and analysis at 0, 1, 3, 5, and 24 h. Thermal control samples, consisting of 500 μL of water fortified with the same amount of stock solution as the samples, were also prepared for extraction and analysis at 0, 1, 3, 5, and 24 h.

Method of Extraction

The analytes were extracted from each 0.5-mL sample of tissue homogenate or plasma by adding a 1-mL aliquot of 60/40 acetonitrile/water with 1% acetic acid. Each vial was sealed with a Teflon-lined cap and the sample mixed on vortex-style mixer for 2 min. The vials were then centrifuged at 1322 g for 10 min and the supernatant transferred to an auto sampler vial for HPLC analysis.

Stability of BPA-Glucuronide in Placental Tissue when produced by In Vivo Phase II Metabolism

Five pregnant (gd 16) female SD rats weighing 295 to 307 g were dosed with ^{14}C-labeled BPA via gavage with a dose of 10 mg ^{14}C-BPA/kg body weight in a stripped corn oil vehicle (Acros Organics, Geel, Belgium, lot #A31). Approximately 550 μCi of radioactivity was administered to each animal. At 15 min after dosing the animals were sacrificed using CO_2 asphyxiation followed by cardiac puncture. The placentas from each animal were collected immediately after sacrifice and pooled as one tissue pool. A homogenate of the pooled placentas consisting of one part tissue and two parts water was prepared by homogenizing on ice for approximately 1 min using a Sonifier Cell Disrupter, model W140 (Misonix Incorporated, Farmingdale, New York). The homogenate was immediately frozen and stored at −80°C until used. Each sample consisted of a 3.0-mL aliquot of tissue homogenate transferred to a 4-mL glass vial. Weighed aliquots of the placenta homogenate were solubilized in Soluene 350 tissue solubilizer (Perkin Elmer Life Sciences, Downers Grove, Illinois). Aliquots of the solubilized homogenate were mixed with Hionic-Fluor liquid scintillation fluid (Perkin Elmer Life Sciences, Downers Grove, Illinois) and the radioactivity determined by LSC. Based on the radioactivity in the placenta homogenate, these samples contained approximately 430 ng-eq BPA/mL. Following transfer to vials, the samples of the homogenate were immediately extracted and analyzed. In addition to the placenta homogenates analyzed at 0 h, additional placenta homogenate samples were prepared for extraction and analysis at 1, 3, 5, and 24 h. A control consisting of placenta homogenate obtained from SD rats that had not received a dose of ^{14}C-BPA was also prepared for extraction and analysis at

0 h; analysis indicated that no background BPA or BPA-G was present in this specimen (data not shown). A fortified control, consisting of placenta homogenate obtained from control SD rats that had not received a dose of BPA and had 100 μL of the stock solution added, was prepared for extraction and analysis at 0 h, as described above. For each time of analysis, duplicate samples were prepared from the pooled SD rat placenta homogenates obtained from rats dosed with ^{14}C-BPA, as well as for the controls and fortified controls.

Determination of the stability of BPA-G in fetal tissue obtained from the animals dosed with ^{14}C-BPA was considered; however, insufficient radioactivity was present in the fetal tissue to produce reliable results. Since ^{14}C-BPA-G was stable at room temperature and neutral pH when added to rat plasma obtained from control animals (see results below), determination of the stability of BPA-G in rat plasma when produced by in vivo phase II metabolism was considered unnecessary.

Methods for Extraction of Placenta Homogenates from Dosed Animals

The analytes were extracted from each 3-mL homogenate sample (1-mL sample for plasma) by adding a 1-mL (0.5 mL for plasma) aliquot of 1% acetic acid in acetonitrile. Each vial was sealed with a Teflon-lined cap and mixed on a vortex-style mixer for 5 min. The vials were then centrifuged at 1322 g for 15 min and the supernatant transferred to an auto sampler vial for HPLC analysis.

Previous work in this laboratory has shown an absolute extraction efficiency of approximately 70% for both ^{14}C-BPA-G and ^{14}C-BPA based on a single solvent extraction of various biological matrices. A 70% extraction efficiency was deemed adequate for this study to determine the change in distribution of ^{14}C-BPA-G/^{14}C-BPA from original ratios because samples were fortified with sufficient ^{14}C-activity levels to ensure reproducible detection of both analytes.

The stock solution used to study stability in biological matrices consisted of 92.2% ^{14}C-BPA-G and 7.8% ^{14}C-BPA when analyzed by HPLC/RAM. When this stock solution was spiked into control placenta homogenate and immediately extracted and analyzed, it showed a distribution of 91.2% ^{14}C-BPA-G and 8.8% ^{14}C-BPA. These data demonstrated that the extraction/analysis technique did not markedly change the composition of the stock solution.

Analytical Methods

The distribution of ^{14}C-BPA-G and ^{14}C-BPA in the sample extracts was determined using HPLC with RAM or fraction collection and LSC. A 50-μL (10 μL for standards) aliquot of sample extract was injected using an Agilent 1100 series G1313A automatic liquid sampler (Agilent Technologies, Wilmington, Delaware) onto a Waters μBondapack C_{18} 3.9 $\times$ 300 mm analytical column (Waters Corporation, Milford, Massachusetts). The sample was separated using a 20-min linear gradient with initial conditions of 95% water, 5% acetonitrile with 0.1% acetic acid (solvent A) ramped to 95% acetonitrile, and 5% water with 0.1% acetic acid (solvent B) with a 5-minute hold at solvent B. The gradient was delivered with an Agilent 1100 series G1312A binary pump at a flow rate of 1 mL/min. The analytes were detected using a Berthold LB507A RAM (Berthold Technologies GmbH, Bad Wildbad, Germany) in the ^{14}C-mode with a 150-uL YiG solid cell. Sample extracts with insufficient ^{14}C-activity for RAM detection were analyzed by collecting 10-sec HPLC fractions from 12 to 18 min into 7-mL scintillation vials (Fisher Scientific, Pittsburgh, Pennsylvania) using a Foxy 200 fraction collector (ISCO Inc., Lincoln, Nebraska). The fractions of eluent collected in each vial then had 6 mL of ULTIMA-FLO M scintillation cocktail added (Packard Instrument Company, Meriden, Connecticut) and were mixed well prior to the determination of radioactivity by LSC. A Beckman LS6000IC (Beckman Coulter Inc., Fullerton, California) liquid scintillation counter was used for the determination of radioactivity.

Data Analysis

The areas under the peaks in chromatograms or reconstructed chromatograms were determined using Turbochrom chromatography software (PE Nelson, San Jose, California). The half-lives for the hydrolysis of BPA-G were calculated as the regression of the semi-logarithmic concentration vs. time using PK Functions (Usansky et al., 2003). Data were expressed as the peak area response for ^{14}C-activity as determined by HPLC/RAM or the radiochromatograms reconstructed from LSC.

RESULTS

Confirmation of the Chemical Identity of Analytes

Identification of BPA-G in the samples analyzed was confirmed using HPLC with atmospheric pressure chemical ionization/mass spectrometry (APCI-MS) in the negative ion mode. The negative ion APCI-MS spectrum showed a base peak at m/z = 403, which corresponds to the $M\text{-}H^-$ pseudo-molecular ion of BPA-G (MW = 404). Previous studies confirm that this spectrum is consistent with mass spectral data from the authentic standard of nonradiolabeled BPA-G (Pottenger et al. 2000). Identification of BPA was confirmed by the HPLC/RAM retention time match with an authentic ^{14}C-BPA standard and by co-chromatography of the authentic ^{14}C-BPA standard with samples of the ^{14}C-BPA-G/^{14}C-BPA standard solution (data not shown).

Chromatography

The HPLC conditions used in this study produced reproducible baseline resolution of ^{14}C-BPA-G and ^{14}C-BPA with retention times of approximately 13 and 15 min, respectively (Fig. 1). Although the use of fraction collection can sometimes reduce chromatographic resolution as compared to detection with the RAM, baseline resolution was maintained between the two analytes using this technique (Fig. 1 and 2). No radiolabled peaks were observed in control extracts (Fig. 3).

BPA-Glucuronide Stability at Various Physicochemical Conditions

The data for BPA-G stability at four different physicochemical conditions are summarized in Table 1. The thermal controls (TC) held at 22°C or 80°C under neutral pH conditions and analyzed at 0 h and 24 h showed no substantial changes in the ratio of BPA-G to BPA. These data indicate that solutions of BPA-G in urine, denatured by the addition of acetonitrile, were stable for up to 24 h when held at neutral pH and either at room temperature (approximately 22°C) or 80°C.

BPA-G was also stable at room temperature up to 24 h when stored in 25/75 (urine/acetonitrile) under acidic conditions (pH 2). However, BPA-G was partially hydrolyzed within 24 h to release the parent aglycone (BPA) (approximately 20% conversion) when stored

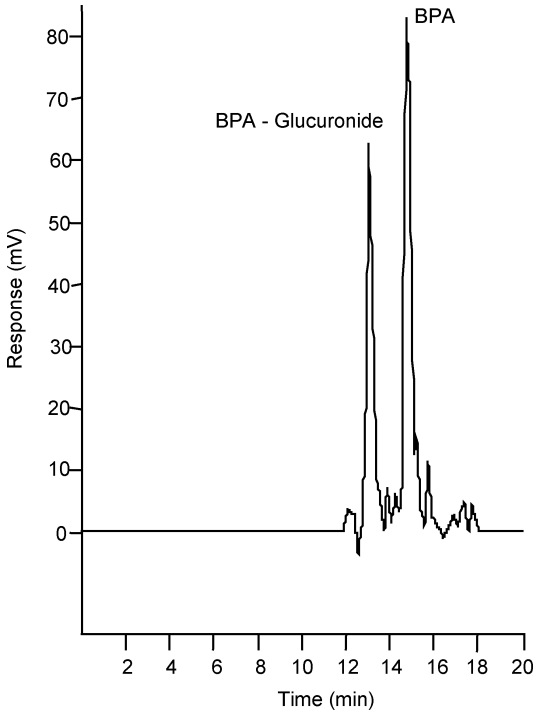

FIGURE 1 **Typical radiochromatogram of fetal homogenate extract from Sprague-Dawley rats receiving an oral dose of ^{14}C-BPA by gavage. Pregnant rats were dosed by gavage on gestation day 16 with a single dose of 10 mg ^{14}C-BPA/kg body weight. Chromatogram was reconstructed from radioactivity determinations in 10s fractions of eluent collected from 12 to 18 min postinjection.**

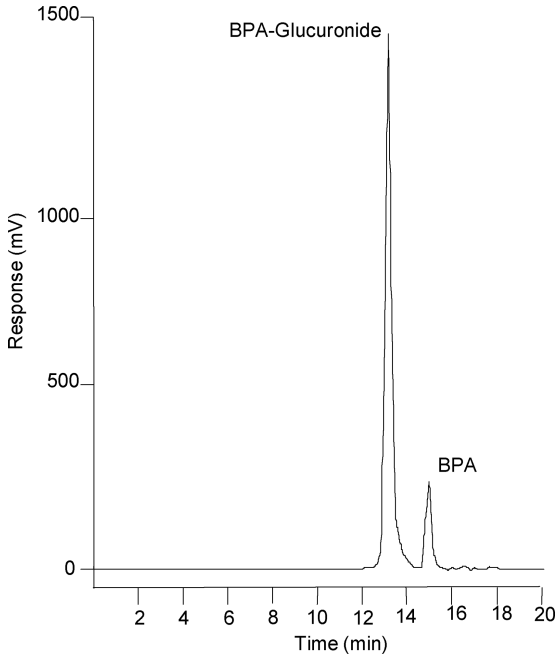

FIGURE 2 **Typical radiochromatogram of control fetal homogenate extract fortified with ^{14}C-BPA-G/^{14}C-BPA. Tissue homogenates prepared from pregnant control rats were fortified with^{14}C-BPA-G/^{14}C-BPA resulting in a sample concentration of 89 ng-eq BPA/mL. Chromatograms were reconstructed from radioactivity determinations in 10s fractions of eluent collected from 12 to 18 min postinjection.**

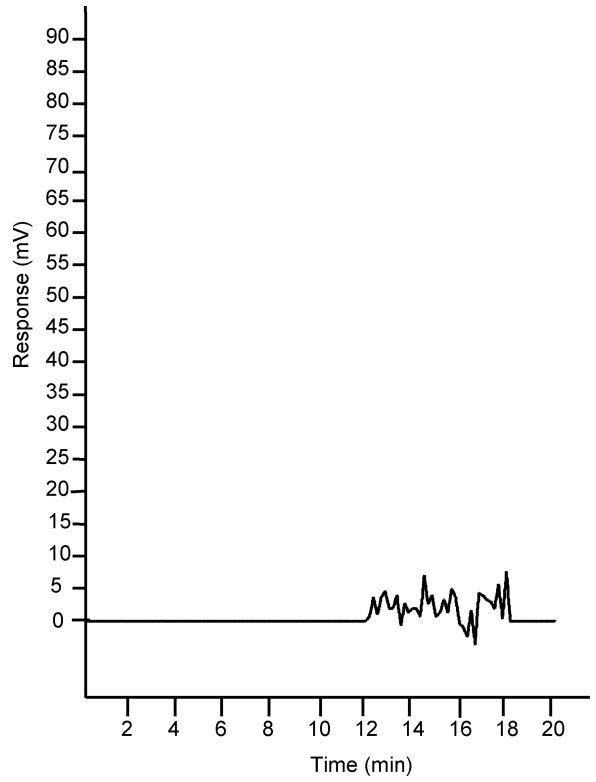

FIGURE 3 **Typical radiochromatogram of control fetal homogenate extract without ^{14}C-BPA-G/^{14}C-BPA fortification. Tissue homogenates prepared from pregnant control rats were analyzed by the same methods as tissues fortified with with^{14}C-BPA-G/^{14}C-BPA. Chromatograms were reconstructed from radioactivity determinations in 10s fractions of eluent collected from 12 to 18 min postinjection.**

at 80°C in 25/75 (urine/acetonitrile) under acidic conditions (pH 2). The half-life for the hydrolysis of BPA-G in these solutions was 106.3 h.

BPA-G was also unstable over a 24-h period when stored in 25/75 (urine/acetonitrile) under basic conditions (pH 9) at either 22°C or 80°C. However, under these conditions, BPA-G was apparently not hydrolyzed to release BPA but was degraded to an unknown compound of greater polarity than BPA-G. Alternately, since BPA-G was decreased, hydrolysis of BPA-G to BPA may have occurred prior to the subsequent conversion to the unknown compound. BPA was no longer detectable in any of the samples adjusted to pH 9, indicating that degradation of BPA also occurred under these conditions.

Stability of BPA-Glucuronide When Added to Biological Matrices Obtained from Control Animals

The data from the experiments studying the stability of BPA-G when added in vitro to biological matrices are shown in Table 2. The data are expressed as the percent of the total radioactivity as either BPA-G or BPA that was found in samples.

TABLE 1 **Stability of BPA-glucuronide and BPA in aqueous/organic solution at four physicochemical conditions**

Physicochemical conditions: temperature, pH	Time to analysis (h)	BPA-G (%)[1]	BPA (%)[1]	Unknown (%)[1]
22°C (TC)[2]	24	95.1	4.9	nd[3]
80°C (TC)	24	94.4	5.6	nd
22°C, pH 2	1	96.7	3.3	nd
	4	94.6	5.4	nd
	8	94.6	5.4	nd
	24	96.2	3.9	nd
80°C, pH 2	1	94.2	5.8	nd
	4	83.3	16.7	nd
	8	72.9	27.1	nd
	24	76.7	23.3	nd
22°C, pH 9	1	87.6	nd	12.4
	4	69.4	nd	30.6
	8	85.9	nd	14.1
	24	86.5	nd	13.5
80°C, pH 9	1	86.9	nd	13.1
	4	77.0	nd	23.0
	8	84.7	nd	15.3
	24	89.1	nd	10.9

[1]Data are the percent of total peak area response for ^{14}C-activity as determined by HPLC/RAM; unknown indicates an unidentified peak, which was degradation product(s) of the analyte(s).

[2]Thermal control (TC) consisted of a sample at neutral pH, held at the temperature specified and analyzed at 0 and 24 h. The percentages of BPA-glucuronide and BPA in the TC at time zero were 95.53 and 4.47, respectively.

[3]nd, indicates not detected; limit of detection was set at 0.5% of the activity injected

The controls (urine/water; 1:5) in these experiments were prepared to determine if BPA-G hydrolysis occurred either nonenzymatically or from any other activity in the urine that was used as the stock solution. These data also allowed for the calculation of the hydrolysis that occurred as a result of activity in the biological matrices (likely enzymatic) vs. any nonspecific hydrolysis (likely nonenzymatic) that occurred in the controls. In the controls, BPA-G was partially hydrolyzed (approximately 8% BPA at 3 h, approximately 30% BPA at 24 h) when stored at neutral pH and room temperature (approximately 22°C). The half-life for the hydrolysis of BPA-G in controls was 50.6 h, demonstrating the instability of

TABLE 2 **Stability of BPA-glucuronide when added to plasma or tissue homogenates from control sprague-dawley rats**

Sample or specimen	Time to analysis (h)	BPA-G (%)[1]	BPA (%)[1]	Corrected BPA-G (%)[2]	Corrected BPA (%)[2]
Control[3]	0	97.1	2.9	na[4]	na
	1	96.7	3.3	na	na
	3	92.0	8.0	na	na
	5	89.6	10.4	na	na
	24	69.7	30.3	na	na
Plasma	0	94.6	5.4	na	na
	1	92.1	7.9	na	na
	3	92.9	7.1	na	na
	5	94.8	5.2	na	na
	24	93.2	6.8	na	na
Placenta	0	90.5	9.5	93.4	6.6
	1	83.4	16.6	86.6	13.4
	3	70.2	29.8	78.2	21.8
	5	57.6	42.4	68.0	32.0
	24	12.0	88.0	42.3	57.7
Fetus	0	93.9	6.1	96.8	3.2
	1	86.0	14.0	89.3	10.7
	3	81.1	18.9	89.1	10.9
	5	67.2	32.8	77.6	22.4
	24	24.5	75.5	54.8	45.2

[1]Data are the percent of total peak area response for ^{14}C-activity as determined by HPLC/RAM.

[2]Data are the percent of total peak area response for ^{14}C-activity corrected for hydrolysis measured in the controls (corrected percent BPA-glucuronide equals 100 minus the corrected percent BPA; corrected percent BPA equals the percent BPA minus the percent control BPA).

[3]Control was 1:5 stock solution:water held at neutral pH and 25°C.

[4]na, not applicable.

BPA-G in diluted mammalian urine under normal room temperature and pH conditions.

BPA-G appeared to be stable in plasma for up to 24 h when held at neutral pH and 22°C. Only very slight variations were observed in the ratio of BPA-G to BPA from 0 to 24 h without further hydrolysis over time as was observed for the controls. Hence, BPA-G under the conditions of this experiment appeared more stable in plasma than in the aqueous solutions.

In the homogenates of placenta, BPA-G was rapidly and extensively hydrolyzed to BPA and glucuronic acid (16.6% BPA at 1 h, 88% BPA at 24 h). The corrected percent of BPA-G and BPA shown in Table 2 are the data from placenta or fetal homogenates corrected for the hydrolysis that occurred in the controls. After subtraction of the percent hydrolyzed in the analogous controls, the values for placenta homogenates were approximately 13% BPA at 1 h and approximately 58% BPA at 24 h; therefore, the biological activity in placenta contributed significantly to BPA-G hydrolysis. The percent BPA released as a result of enzymatic activity can be calculated by dividing the corrected BPA (column 6, Table 2) by the total BPA released (column 4, Table 2) and multiplying by 100. Clearly, enzymatic activity in placenta homogenates was responsible for the majority of the BPA-G hydrolysis, ranging from 66% to 81% of the total amount of BPA produced. The hydrolysis of BPA-G by placenta homogenates was significantly faster than the hydrolysis of BPA-G that occurred in the controls at all times postfortification with BPA-G. The half-life for the hydrolysis of BPA-G in placental homogenates was calculated to be 8.3 h as compared to 50.6 h for controls (Table 2 and Fig. 4).

Hydrolysis of BPA-G by fetal homogenates occurred more slowly than in the placenta homogenates with a half-life of 12.6 h (Table 2 and Fig. 4). Nevertheless, the biological activity in fetal homogenates was responsible for the majority of the BPA produced from BPA-G, ranging from 52% to 76%. The corrected BPA-G

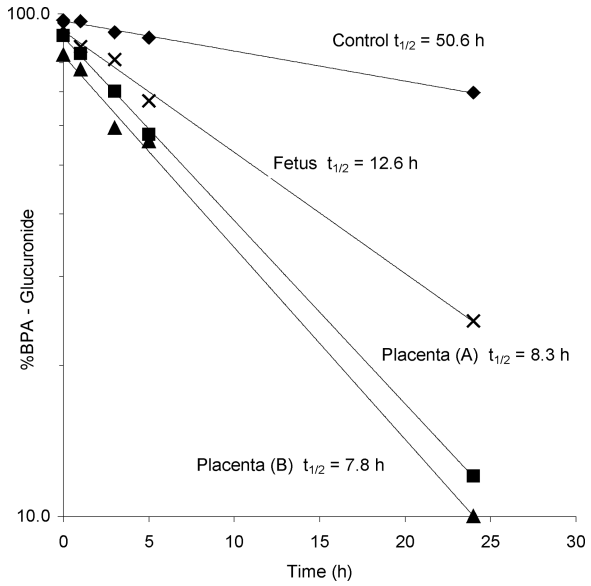

FIGURE 4 **Rate of hydrolysis of BPA-glucuronide in tissue homogenates over time when stored at room temperature. Control samples were 500 μL of water fortified with the same amount of ^{14}C-BPA-G/^{14}C-BPA stock solution as were the placenta homogenates from control rats. Fetus is the data from control rat fetal homogenates with ^{14}C-BPA-G/^{14}C-BPA stock solution added. Placenta (A) is the data from control rat placenta homogenates with ^{14}C-BPA-G/^{14}C-BPA stock solution added. Placenta (B) is the data from placenta homogenates derived from rats receiving ^{14}C-BPA by oral gavage.**

hydrolysis to BPA and glucuronic acid by rat fetal homogenates held at neutral pH and room temperature was approximately 11% BPA at 1 h and about 45% BPA at 24 h.

Stability of BPA-Glucuronide in Placenta when produced by In Vivo Phase II Metabolism

The stability of BPA-G in placenta homogenates when generated via phase II metabolism in vivo is shown in Table 3 and Figure 4. ^{14}C-BPA-G and ^{14}C-BPA were the only radiolabeled peaks observed in this homogenate following analysis by HPLC. Replicate analyses showed generally good reproducibility with somewhat greater variability observed at 3 and 24 h (12.4% and 4.1% difference between replicate analyses, respectively).

The pooled (n = 5) placenta homogenates from rats dosed with ^{14}C-BPA also readily hydrolyzed BPA-G to release BPA. The approximate average of the replicate determinations of BPA-G and BPA were approximately 86% and 14% at 0 h and about 40% and 60% at 24 h, when corrected for the hydrolysis that was observed in controls. Similar to that observed when placental homogenates were prepared from controls and fortified with ^{14}C-BPA-G, the half-life for hydrolysis of BPA-G was 7.8 h (Fig. 4).

DISCUSSION

At either acidic or neutral pH at room temperature (approximately 22°C), BPA-G was generally stable in 25/75 urine/acetonitrile for up to 24 h. However, at 80°C and acidic conditions, hydrolysis of BPA-G began

TABLE 3 **Stability of BPA-glucuronide in placenta homogenates pooled from five Sprague-Dawley rats receiving a single oral dose of ^{14}C-BPA**

Time to analysis (h) and replicate identification	BPA-G (%)[1]	BPA (%)[1]	Control BPA (%)[1,3]	Corrected BPA-G (%)[2]	Corrected BPA (%)[2]
0—A	84.4	15.6	2.9	87.3	12.7
0—B	81.2	18.8	2.9	84.1	15.9
1—A	77.6	22.4	3.3	80.9	19.1
1—B	77.3	22.7	3.3	80.5	19.5
3—A	65.5	34.5	8.0	73.5	26.5
3—B	53.1	46.9	8.0	61.1	38.9
5—A	57.3	42.7	10.4	67.7	32.3
5—B	54.0	46.0	10.4	64.4	35.6
24—A	8.0	92.0	30.3	38.3	61.7
24—B	12.1	87.9	30.3	42.4	57.6

[1]Data are the percent of total peak area response for ^{14}C-activity as determined by HPLC/RAM or radiochromatogram reconstructed from LSC and are the mean of duplicate analyses done for each replicate.

[2]Data are the percent of total peak area response for ^{14}C-activity corrected for hydrolysis measured in the controls (corrected percent BPA-glucuronide equals 100 minus the corrected percent BPA; corrected percent BPA equals the percent BPA minus the percent control BPA).

[3]Data are the hydrolysis of BPA-G in diluted urine from Table 2.

within 1 h. Adjusting the urine acetonitrile solution to basic conditions (pH of 9) may have resulted in the hydrolysis of BPA-G to BPA with subsequent degradation of BPA-G and BPA to a compound more polar than BPA. Alternately, the BPA-G may have been degraded to the more polar compound with hydrolysis of the glucuronic acid moiety. This degradation occurred within 1 h at both 22°C and 80°C. These data show that extraction procedures employing high pH are likely to result in the inaccurate determination of both BPA-G and BPA concentrations. Acidic extraction conditions at elevated temperatures (80°C) will also affect the accuracy of both BPA and BPA-G determinations and should be avoided. These data also suggest that sample extracts prepared for BPA analysis that are likely to also contain BPA-G should either be analyzed immediately or stored at −80°C until analysis.

The hydrolysis of BPA-G in the control samples from Table 2 (5:1 dilution of urine stock solution with water) was likely mediated by a direct chemical (nonenzymatic mechanism) reaction of the BPA-G with water. Hydrolysis from enzymatic activity was not likely since only very slight traces of protein are typically excreted via the urine. Since the controls in Table 2 were a mixture of water and urine, these data also indicate that BPA-G would not be stable in urine at neutral pH and room temperature. Under these conditions about an additional 5% of the BPA-G in these urine specimens was hydrolyzed within 3 h. The sole study on the pharmacokinetics of BPA in humans indicated that only BPA-G was found in the urine (Völkel et al. 2002). Hence, any prior studies that stored or held human urine at room temperature for even brief periods may have erroneously reported the presence of BPA in urine.

The addition of acetonitrile to the stock solution of ^{14}C-BPA-G/^{14}C-BPA in urine resulted in greater stability of BPA-G at room temperature and neutral pH (Table 1, TC data). This greater stability was likely the result of decreasing the accessibility (and hence the rate of reaction) of BPA-G with water by the 3:1 dilution with acetonitrile, rather than denaturing any biological hydrolytic activity in the urine.

Contrasting with the results following dilution of the stock solution 5:1 with water, the ^{14}C-BPA-G was stable in plasma for up to 24 h (Table 2). Since blood plasma is about 90% water (Banks 1986), ample water was available for reaction with BPA-G. The stability of BPA-G in blood plasma may be a result of the weak binding of BPA-G with plasma proteins resulting in the sequestration of this material from reaction with the water. Support for this explanation is found in the extensive binding of BPA (approximately 95%) to plasma proteins (Mayersohn 2003). These data suggest that plasma, as a biological matrix, is less likely to yield artificially high free BPA levels as an artifact of inappropriate sample handling. However, extraction techniques for plasma or blood employing low pH and high temperatures could still result in erroneously high BPA values.

The addition of ^{14}C-BPA-G to homogenates of placenta or fetal tissue resulted in about 10% to 13% conversion of the BPA-G to free BPA within 1 h. Within 24 h, free BPA represented 88% and 75.5% of the test material present in placenta or fetal homogenates, respectively (Table 2). These data are not corrected for the nonenzymatic hydrolysis that was observed in the controls. Regardless of the mechanism of hydrolysis, these data demonstrate that without appropriate conditions for specimen collection, preparation, and analysis, these biological matrices may yield artificially high free BPA levels. The concentration of BPA-G used in these experiments (89 ng-eq BPA/mL) was in the same range of the BPA-G concentrations observed in these tissues following a 10 mg/kg oral dose of ^{14}C-BPA to pregnant rats. Thus, the measurement of BPA-G stability in this range was representative of actual tissue concentrations of this metabolite resulting from an in vivo administration of BPA. The corrected values for the percent of BPA-G hydrolyzed to BPA in these tissue homogenates was most likely to due to activity of β-glucuronidase in mammalian placenta (Christie 1968; Blumer 1967) and fetus (Lucier et al. 1977; Harris et al. 1991).

The placenta homogenate from rats dosed with ^{14}C-BPA showed hydrolysis of BPA-G to BPA of approximately 90% within 24 h (or about 60% when corrected for nonenzymatic activity observed in the controls) (Table 3). This was similar to the hydrolysis observed when BPA-G was added to placenta homogenates from control rats where hydrolysis of BPA-G to BPA was approximately 88% (or about 58% when corrected for hydrolysis in controls). The similar hydrolysis rates of BPA-G to BPA in the placenta homogenate from control rats as compared to the placenta homogenates from rats dosed with ^{14}C-BPA shows that the BPA-G generated by in vivo metabolism is not uniquely stable

(Fig 4). Thus, any of the conditions shown to affect the hydrolysis or degradation of BPA-G or BPA in this study should produce similar effects in biological specimens where these analytes are produced as a result of in vivo exposures.

To further investigate the role of enzymatic hydrolysis vs. nonenzymatic hydrolysis of BPA-G in mammalian tissues, additional experiments could be conducted using β-glucuronidase inhibitors. Such studies could examine the stability and/or hydrolysis of BPA-G in tissues after pretreatment of animals with a variety of compounds reported to be β-glucuronidase inhibitors such as N-cyclohexyl-5-O-acetyl-2,4-O-p-methoxybenzylidene) -D-glucaro-1-amide-6,3-lactone (Takada et al. 1982), ganoderenic acid A (Kim et al. 1999), or saccharolactone (Joyce et al. 1986).

The time required to conduct this study also allowed for the determination of storage stability at -80°C for BPA-G in rat placenta homogenates over 78 days. Initial analysis of the individual animals specimens at 0 h showed an average of approximately 22% BPA and 88% BPA-G. In another study, pooled placenta homogenates analyzed following 78-day storage at -80°C, showed approximately 17% BPA (data not shown). These data indicate that no significant hydrolysis of BPA-G to BPA occurred in rat placenta homogenates under these storage conditions. Similar storage stability would be likely for BPA-G in other tissues stored under these conditions.

Based on prior studies of the pharmacokinetics of BPA, BPA-G was always found in significantly greater concentrations in the biological matrices analyzed as compared to BPA. Therefore, caution must be taken when collecting, storing, preparing, and analyzing biological specimens for BPA and/or BPA-G as hydrolysis may occur leading to erroneous conclusions regarding the free parent that was actually present. Similarly, caution should be used in the interpretation of studies analyzing human tissues that have significant glucuronidase activity such as placenta as even brief periods of storage or processing at room temperature may result in significant BPA-G hydrolysis.

The results of the current study support our hypothesis that BPA-G instability during specimen collection and analysis contributes to the presence of free BPA in the biological samples. Furthermore, these data demonstrate the importance of fully validating any sample collection, extraction, storage, and analysis procedures with the appropriate matrix spikes and controls. The importance of appropriate matrix spikes and controls is twofold. First, they ensure the accuracy of the data generated, and second, they can assist in troubleshooting the source of erroneous data to specific steps or techniques used in the method. Matrix spikes and controls may take many forms based on the requirements of the study. However, at a minimum, they must account for each step in the method that has the potential to introduce error into the analytical determination. In the case of methods for BPA analysis in specimens where BPA-G may also be present, the stability of BPA-G in the matrix of interest under the conditions of analysis should be determined with appropriate spikes and controls with authentic BPA-G. Without this approach, accuracy in the determination of BPA-G and/or BPA cannot be certain. Analytical methods that directly or indirectly account for BPA and its metabolites provide a more complete understanding of BPA metabolism and disposition and can serve to cross-verify the data generated. Therefore, methods that incorporate the direct determination of both BPA-G and BPA or account for parent compound vs. total BPA via analysis prior to and after hydrolysis would have an advantage over methods that analyze for a single analyte.

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