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Characterization of New Bisphenol A Metabolites Produced by CD1 Mice Liver Microsomes and S9 Fractions

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Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA) is a widely used industrial chemical resulting in occupational and consumer exposure. BPA possesses weak estrogenomimetic activity and can be cytotoxic, though the underlying mechanisms of its toxicity toward cells are not completely understood. The metabolism of BPA by CD1 mice liver microsomal and S9 fractions was investigated. Nine metabolites were isolated and characterized using HPLC and mass spectrometry. Many of these metabolites were characterized for the first time in mammals, namely isopropyl-hydroxyphenol (produced by the cleavage of BPA), a bisphenol A glutathione conjugate, glutathionyl-phenol, glutathionyl 4-isopropylphenol, and BPA dimers. Most of these metabolites apparently share a common metabolic pathway, for which considerable evidence supports the hypothesis of the production of a reactive intermediate, and also helps explain BPA cytotoxicity.

KEYWORDS: BPA; in vitro metabolism; mouse; endocrine disruptor

INTRODUCTION

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane, CAS Registry No. 80-05-7] (BPA) is used as a monomer in the production of epoxy-phenolic resins, polycarbonate plastics, and as an antioxidant in PVC plastics. BPA-based polymers are widely used in industry for the packaging of food (lacquered coating of food cans) and drinks (polycarbonate bottles). These polymers can be a source of contamination of human foodstuff (*1*, *2*). It has been demonstrated that BPA monomers can be released from packaging material and then migrate into foods and beverages (*3*, *4*). The rate of migration is enhanced by treatments such as heat processing (*5*, *6*). Human exposure to BPA is expected to be relatively low but nevertheless daily, and the presence of BPA in human biological fluids has already been demonstrated (*7*−*9*).

BPA can induce oxidative stress (*10*, *11*) and can be chemically converted into reactive intermediates, presumably via a cytochrome P450 (CYP) pathway (*12*, *13*). These intermediates have been shown to bind DNA in vitro and in vivo *(12, 13)*. BPA was also reported to be a synthetic estrogenic agent (*14*). BPA estrogenic activity is weak compared with 17-β-oestradiol, but this chemical has been shown to efficiently bind and activate human estrogen receptors (*5*, *15*). According to Yoshihara (*16*), the estrogenicity of BPA is enhanced through its biotransformation by rat liver S9 fractions. Thus, understanding the metabolism of BPA may be of great importance when attempting to assess its toxicity at the cellular level, as well as when assessing its pharmacological properties.

Several studies on the biotransformations of BPA have been carried out in vivo (*17*−*21*) and in vitro (*16*, *22*−*27*). Bisphenol A glucuronide was characterized as the major metabolite of BPA. Other metabolites such as BPA sulfate conjugate, BPA diglucuronide, 5-hydroxybisphenol A, and the corresponding sulfate conjugate were also identified. Recently, an extensive study of BPA metabolism in pregnant CD1 mice demonstrated the formation of additional metabolites (*28*) showing that the metabolism of BPA was even more complex. To elucidate the mechanism of its toxicity, more extensive knowledge of BPA biotransformation is essential.

In the present study, the biotransformation of radiolabeled BPA was investigated in vitro using liver microsomes and S9 systems prepared from female and male CD1 mice. Metabolites were separated, isolated by high-performance liquid chromatography (HPLC), and analyzed by mass spectrometry (MS), MS^n, and/or LC-MS.

MATERIALS AND METHODS

Chemicals. ^{3}H-labeled bisphenol A [([^{3}H]BPA); 2,2-bis(4-hydroxyphenyl)propane], with a specific activity of 185 GBq/mmol, was purchased from Moravek Biochemicals (Mercury Lane, Brea, CA) and stored in ethanol at −20 °C. In these conditions only limited tritium exchange occurs (< 3%/6 months). Prior to the experiments, the solution was evaporated to dryness under a nitrogen stream and was redissolved in ethanol. Its purity was verified by radio-HPLC and was greater than 99.9%.

Methanol and acetonitrile were purchased from Scharlau Chemie S.A. (Barcelona, Spain); ethanol and acetic acid were purchased from

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Merck (Briare-Le-Canal, France). Unlabeled BPA and all biochemical reagents were purchased from Sigma Aldrich (L'Isle d'Abeau Chesnes, France).

Animals. A total of 10 female and 10 male CD1 mice (16−20 g) were obtained from Harlan France SARL (Gannat, France). The animals were housed at 21 ± 1 °C with a relative humidity of 60−70% under a 12 h light/dark cycle. They were fed a standard commercial diet (UAR, Villemoisson-Sur-Orge, France) and were allowed free access to water.

Preparation of Mice Liver Subcellular Fractions. Mice were killed by cervical dislocation followed by exsanguination and their livers were perfused immediately using 0.1 M sodium phosphate buffer (pH 7.4). Livers from two animals were pooled and weighed after homogenization by a Potter-Elvehjem glass Teflon homogenizer in 4 volumes/g of ice-cold phosphate buffer. S9 fractions were obtained by centrifugation at 10000*g* for 20 min at 5 °C. Cytosol and microsomal pellets were obtained by centrifugation of S9 fractions at 105000*g* for 60 min. Microsomes were resuspended with gentle homogenization in 1 volume/g of 0.1 M phosphate buffer containing 20% glycerol. S9 fractions, microsomes, and cytosols were stored at −80 °C until use. The protein content of subcellular fractions was determined by the method of Lowry (*29*).

Metabolism of [^{3}H]BPA by Mice Liver S9 and Microsomes. In vitro biotransformations of BPA were studied by incubating for 2 h at 37 °C under shaking radiolabeled [^{3}H]BPA (8400 Bq) fortified with unlabeled BPA at different concentrations (20, 50, 100, 200, and 500 μM), with 2 mg of microsomal protein or 8 mg of S9 protein (100 mg liver equivalent) in a final volume of 1 mL of 0.1 M sodium/potassium buffer, 5 mM $MgCl_2$ (pH 7.4) containing an NADPH generating system (NADP 1.3 mM, glucose-6-phosphate 5 mM, glucose-6-phosphate dehydrogenase 2 IU). All incubations were carried out in triplicate.

Blank samples without an NADPH generating system were used as controls for non-NADPH-mediated BPA transformation.

Reactions were stopped by adding 3 volumes of methanol and samples were centrifuged for 10 min at 6000*g*. Supernatant samples (500 μL) were concentrated under a nitrogen stream and analyzed by HPLC coupled to online radioactivity detection.

β Glucuronidase Hydrolysis Assays. For the confirmation of BPA glucuronide structure, extracts of S9 incubation media were incubated in 0.1 M sodium acetate buffer (pH 5) for 4 h at 37 °C, with 500 IU of bovine liver β glucuronidase (controlled without sulfatase activity using 4-nitrophenyl sulfate as substrate) prior to analysis by radio-HPLC.

In Vitro Synthesis of [^{3}H]BPA Glucuronide and [^{3}H]BPA Sulfate. [^{3}H]BPA glucuronidation was performed by incubating 2 mg microsomal protein with labeled BPA (100 μM; 8400 Bq) and UDPGA (1 mM) in 1 mL 0.5 M Tris buffer (pH 7.4) containing 10 mM $MgCl_2$, for 2 h at 37 °C.

[^{3}H]BPA sulfate was produced by incubating 2 mg of cytosolic protein with [^{3}H]BPA (100 μM; 400 Bq) and 3′-phosphoadenosine-5-phosphosulfate (PAPS) 40 μM in a final volume of 1 mL of Tris buffer 50 mM (pH 7.5), 5 mM $MgCl_2$, for 2 h at 37 °C.

Synthesized [^{3}H]BPA glucuronide and BPA sulfate were analyzed by HPLC, characterized by MS and used as standards.

Apparatus. Samples were analyzed by HPLC on an HP 1050 apparatus (Hewlett-Packard, Waldbronn, Germany) equipped with a Rheodyne model 7125 injector (Rheodyne, Cotati, CA) connected for radioactivity detection to a Radiomatic flo-one/β A 500 instrument (Radiomatic, La-Queue-Lez-Yvelines, France) using Flow-scint II as the scintillation cocktail (Packard Instrument Co., Rungis, France) in order to establish metabolic profiles, or to an HP 1050 UV detector set at 270 nm and a Gilson model 201/202 fraction collector (Gilson France, Villiers-Le-Bel, France) for metabolite isolation.

Mass spectrometry analyses were performed using a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, Les Ullis, France) equipped with an electrospray ionization (ESI) source. Typical conditions for recording mass spectra were as follows: spray voltage, 4 kV; capillary voltage, −22 V; capillary temperature, 200 °C. All mass spectra were acquired using automatic gain control conditions and the helium buffer gas was used as the collision gas for MS^n studies. Sample solutions (typically 1 ng/μL in methanol−water (50:50, v/v) were infused at a flow rate of 3 μL/min into the ionization source. Hydrogen/Deuterium exchange experiments were carried out using CH_3OD and D_2O as solvents, using the same operating conditions.

HPLC Analysis and Metabolite Quantification. The HPLC system consisted of a Zorbax C18 column (250 × 4.6 mm, 5 μm) (Interchim, Montluçon, France) coupled to a Kromasil C18 guard precolumn (18 × 4.6 mm, 5 μm) (Interchim). Mobile phases were A: ammonium acetate buffer (20 mM, pH 3.5)/acetonitrile (95:5; v/v) and B: ammonium acetate buffer (20 mM, pH 3.5)/acetonitrile (10:90; v/v). The flow rate was 1 mL/min and the temperature was maintained at 30 °C. A 5-step gradient was used as follows: 0−4 min 100% A; 4−6 min linear gradient from 100% A to A:B 85:15 v/v, 6−16 min A:B 85:15 v/v; 16−18 min linear gradient from 15% B to 25% B; 18−28 min A:B 75:25 v/v; 28−30 min linear gradient from 25% B to 30% B; 30−37 min A:B 70:30 v/v; 37−39 min linear gradient from 30% B to 70% B; 39−50 min A:B 30:70 v/v; 50−52 min linear gradient leading to 100% B; 52−62 min 100% B.

[^{3}H]-BPA and related metabolites were monitored by online radioactivity detection (Flo-one β A500, Packard) and quantified by integrating the area under detected peaks.

Metabolite Isolation. Additional incubations of 50 μM BPA (1 700 Bq) were carried out in the same conditions as described above with liver microsomes and liver S9 fractions, respectively, in order to obtain enough material for metabolite production.

After the addition of 3 volumes of methanol and centrifugation, supernatants were individually concentrated under a nitrogen stream and the metabolites were separated by HPLC using the analytical system described above. The collected fractions corresponding to the different BPA metabolites were individually evaporated to dryness under a nitrogen stream, then dissolved in 5 mL ammonium acetate buffer (50 mM, pH 3.2) and extracted using an Oasis HLB 0.2 g LP cartridge (Waters, Milford, MA) previously washed with methanol (5 mL) and equilibrated with the ammonium acetate buffer (5 mL). Elution was performed successively with 5 mL of the buffer and with 5 mL of methanol (3 times). The methanol eluate was evaporated to dryness under a nitrogen stream and subjected to structural analysis.

Combined High Performance Liquid Chromatography−Mass Spectrometry (LC-MS). For LC-MS experiments, the LCQ mass spectrometer was fitted with an atmospheric pressure chemical ionization (APCI) source operated in the negative ion mode. Liquid chromatography was carried out using a Thermo Separation P4000 pump (Thermo Finnigan, Les Ullis, France) and a Thermo Separation AS3000 autosampler was used for sample injection. The LC column used was a Zorbax SB-C18 column (250 × 4.6 mn, 5 μm) from Agilent Technologies (Les Ullis, France). Separation was achieved at a flow rate of 1 mL/min using the following gradient elution: 100% A from 0 to 4 min, 100% A to 100% B from 4 to 39 min, and then 100% B from 39 to 50 min, with A, water−acetonitrile (95:5; v/v) and B, water−acetonitrile (10:90, v/v).

Operating conditions of the APCI source were as follows: vaporizer temperature, 450 °C, capillary temperature, 150 °C, capillary voltage, −20 V. Ion isolation and collision conditions were adjusted separately for each metabolite in order to gain maximum structural information.

Statistical Analysis. Mean values were compared by two-tailed Student's *t* test and the differences were considered to be significant when $P < 0.05$.

RESULTS

Metabolism of BPA by Mice Liver Microsomes. A representative radio-chromatogram corresponding to the analysis of microsomal incubation supernatants is given in **Figure 1**, showing nine different radioactive peaks.

The radioactive peak corresponding to unchanged BPA (R_T: 42.80 min) accounted for 60% to 80% of the radioactivity incubated. In our HPLC system, seven compounds eluted before BPA (R_T: 10.50, 12.9, 13.80, 20.80, 22.60, 23.40, and 36.20 min, respectively), whereas two metabolites (R_T: 45.00 and 46.30 min, respectively) exhibited a lower polarity than BPA.

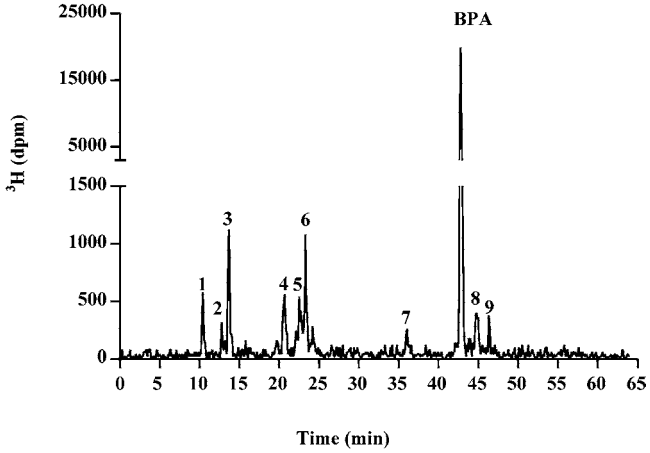


Figure 1. Representative HPLC-radiochromatogram obtained after incubation of BPA (100 μM, 2 h, 37 °C) with CDI mice liver microsomes.

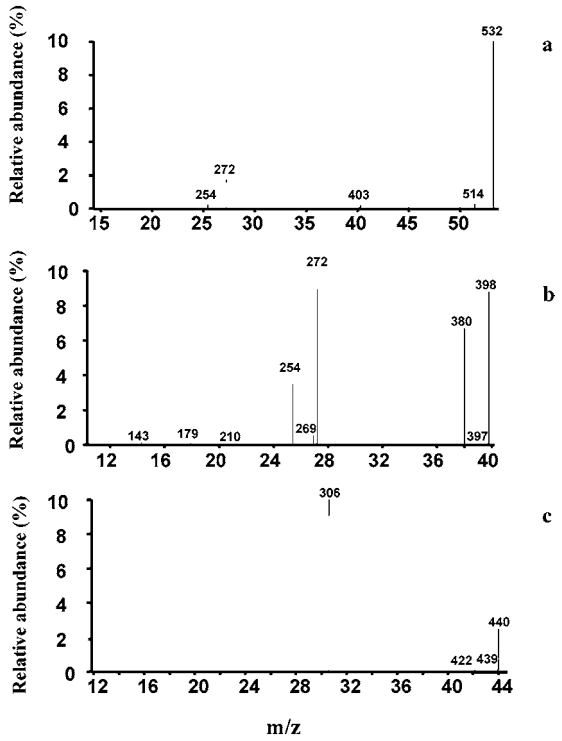


Figure 2. ESI-MS^2 analysis of (**a**) glutathione BPA conjugate (peak 6), (**b**) glutathionyl-phenol (peak 1), and (**c**) glutathionyl 4-isopropylphenol (peak 2).

Polar metabolites (peaks 1−7) were isolated as described in the materials and methods section and their structures were investigated by mass spectrometry using electrospray in the negative ionization mode (**Table 1**). The ESI analysis of the metabolite corresponding to peak 6 gave an $[M - H]^-$ ion at *m*/*z* 532 (**Figure 2a**).

The fragmentation of this ion (MS^2) led to the formation of the *m*/*z* 272 ion resulting from the cleavage of the glutathione thioether bond, and the *m*/*z* 403 ion resulting from the loss of glutamic acid. Thus, this metabolite was characterized as a glutathione conjugate of bisphenol A. The mass spectrometric analysis of the metabolite corresponding to peak 1 (**Figure 2b**) gave an $[M - H]^-$ ion at *m*/*z* 398, which yielded *m*/*z* 272 and *m*/*z* 269 fragment ions in MS^2. These fragments corresponded to the same decomposition pathways as observed for peak 6, and thus metabolite 1 was identified as glutathionyl phenol. The metabolite obtained from peak 2 exhibited an $[M - H]^-$ ion at *m*/*z* 440 (**Figure 2c**) and was fragmented in MS^2 to *m*/*z* 306 (depronated glutathione). In MS^3, this fragment produced an $[M - H]^-$ ion at *m*/*z* 272 (**Table 1**). Thus, this metabolite was identified as glutathionyl 4-isopropylphenol.

The three metabolites, with respective retention times of 13.8, 22.6, and 36.2 min were identified as isopropyl-hydroxyphenol, 2,2-bis(4-hydroxyphenyl)-1-propanol, and 5-hydroxybisphenol A (BPA catechol) on the basis of MS analyses (**Table 1**).

The metabolite with a R_T of 20.80 min could not be identified by MS analysis because of insufficient material, but this metabolite coeluted with authentic phenol.

The metabolites eluting in our chromatographic system after unchanged BPA (peaks 8 and 9, **Figure 1**), collected and analyzed by LC/ESI-MS, gave similar results (**Figure 3**).

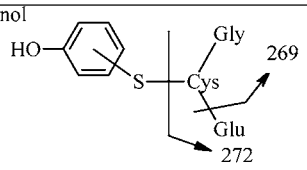
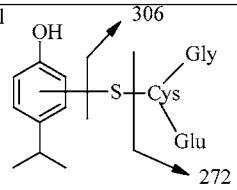
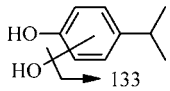
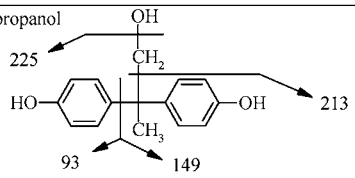
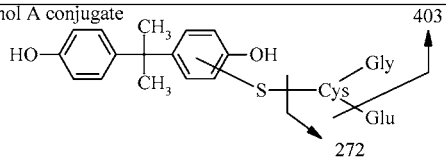
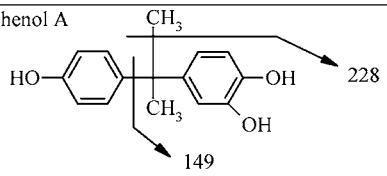
In both cases, two $[M - H]^-$ quasimolecular ions were observed: a major one at *m*/*z* 453 and another at *m*/*z* 451. The fragmentation of *m*/*z* 453 ion in MS^2 produced fragment ions at *m*/*z* 359 and 319 (see **Figure 3**). In MS^3, the *m*/*z* 359 ion gave a fragment ion at *m*/*z* 265 (corresponding to the loss of the phenol group), and the *m*/*z* 319 ion gave a major fragment ion at *m*/*z* 225 (also corresponding to the loss of a phenol group). These results support the hypothesis of the formation of BPA dimers by linkage of two BPA molecules, thus possessing a molecular weight of 454 and detected as $[M - H]^-$ quasi-molecular ions at *m*/*z* 453. These metabolites are fragmented in MS^2, either by the loss of an isopropyl-phenol moiety (*m*/*z* 319), or by the loss of a phenol moiety (*m*/*z* 359), as shown in **Figure 4**.

Both resulting fragment ions can be further fragmented in MS^3, as detailed above. Further structural characterization of metabolites 8 and 9 by NMR analysis was not possible due to the limited amount of metabolites available. However, these metabolites were analyzed by APCI-MS, and similar results were obtained (data not shown). When infused into the ESI source in CH_3OD/D_2O, the quasimolecular species was detected at *m*/*z* 456, corresponding to an $[Md_4\text{-}D]^-$ ion, thus indicating the presence of 4 exchangeable hydrogen atoms in the dimeric structure. These results indicate that for metabolite 8 as well as for metabolite 9, the dimerization of BPA does not involve the phenolic groups via ether bonds. Consequently, metabolites 8 and 9 should correspond to the two possible isomers resulting from the carbon−carbon linkage of two molecules of BPA, as proposed in **Figure 4**. When metabolites 8 and 9 were analyzed by ESI-MS, we observed an additional $[M - H]^-$ quasi-molecular ion at *m*/*z* 451, whose fragmentation in MS^2 led to a major fragment ion at *m*/*z* 436 (corresponding to the loss of a methyl radical), *m*/*z* 357 (corresponding to the loss of a phenol group) and to two minor fragment ions at *m*/*z* 225 (likely due to the cleavage of the two ether bonds of such dimeric parent ions). Thus, *m*/*z* 451 was tentatively identified as a cyclic dimer of BPA, which would be coeluted from the HPLC column with metabolites 8 and 9. Three isomers could theoretically be formed, one of which is shown in **Figure 4**.

When BPA was incubated with female and male mice liver microsomes in triplicate for 2 h at 37 °C in the presence of NADPH, no sex-related difference was observed. In addition no metabolites were formed when an NADPH generating system was omitted from the incubation mixtures.

Metabolite formation rates were investigated over a substrate concentration range of 20−500 μM. The formation of the two major hydroxylated metabolites, namely isopropyl-hydroxy-phenol and 5-hydroxybisphenol A (respectively peaks 3 and 7, **Figure 1**) as a function of BPA concentration is shown in **Figure 5** and followed Michaelis−Menten kinetics (the apparent

Table 1. Mass Spectrometric Data and Structure of BPA Metabolites 1–7

[peak number] Rt (min)	Name and Structure	$[M-H]^-$ (m/z)	MS^2
[1] 10.5	Glutathionyl-phenol 	398	272
[2] 12.9	Glutathionyl 4-isopropylphenol 	440	306 272 (MS^3)
[3] 13.8	Isopropyl-hydroxyphenol 	151	133
[4] 20.8	Not Identified		
[5] 22.6	2,2-bis-(4-hydroxyphenyl)1-propanol 	243	225 213 149 93
[6] 23.4	Glutathione bisphenol A conjugate 	532	403 272
[7] 36.2	5-hydroxy bisphenol A 	243	228 149

$K_m = 84.7 \pm 31.7$ μM and the apparent $V_m = 1.96 \pm 0.4$ nmol/mg/h for isopropyl-hydroxyphenol, the apparent $K_m = 837 \pm 310$ μM and the apparent $V_m = 3.27 \pm 0.67$ nmol/mg/h for 5-hydroxybisphenol A). Whereas the production of the two metabolites eluted after BPA (respectively peaks 8 and 9, **Figure 1**) did not follow Michaelis−Menten kinetics but was linear up to 500 μM BPA (**Figure 6**).

Metabolism of BPA by Mice Liver S9 Fractions. BPA incubations (20−500 μM) were carried out with S9 fractions of female and male mice livers (2 h, 37 °C) in the presence of NADPH. Each incubation was carried out in triplicate, with three different S9 fractions. No significant difference was observed between females and males (data not shown). Radio-HPLC analyses of the supernatants of S9 BPA incubations (**Figure 7**) showed high metabolization rates, compared with microsomes; unchanged BPA accounting for 10 to 50% of the total radioactivity. Metabolites eluted at respective R_T of 10.5, 12.9, 13.8, 23.4, and 24.2 min were detected.

All metabolites were collected and analyzed by mass spectrometry using negative electrospray ionization mode, some of them were found to be similar to metabolites already characterized from the supernatants of microsomal incubations: gluta-

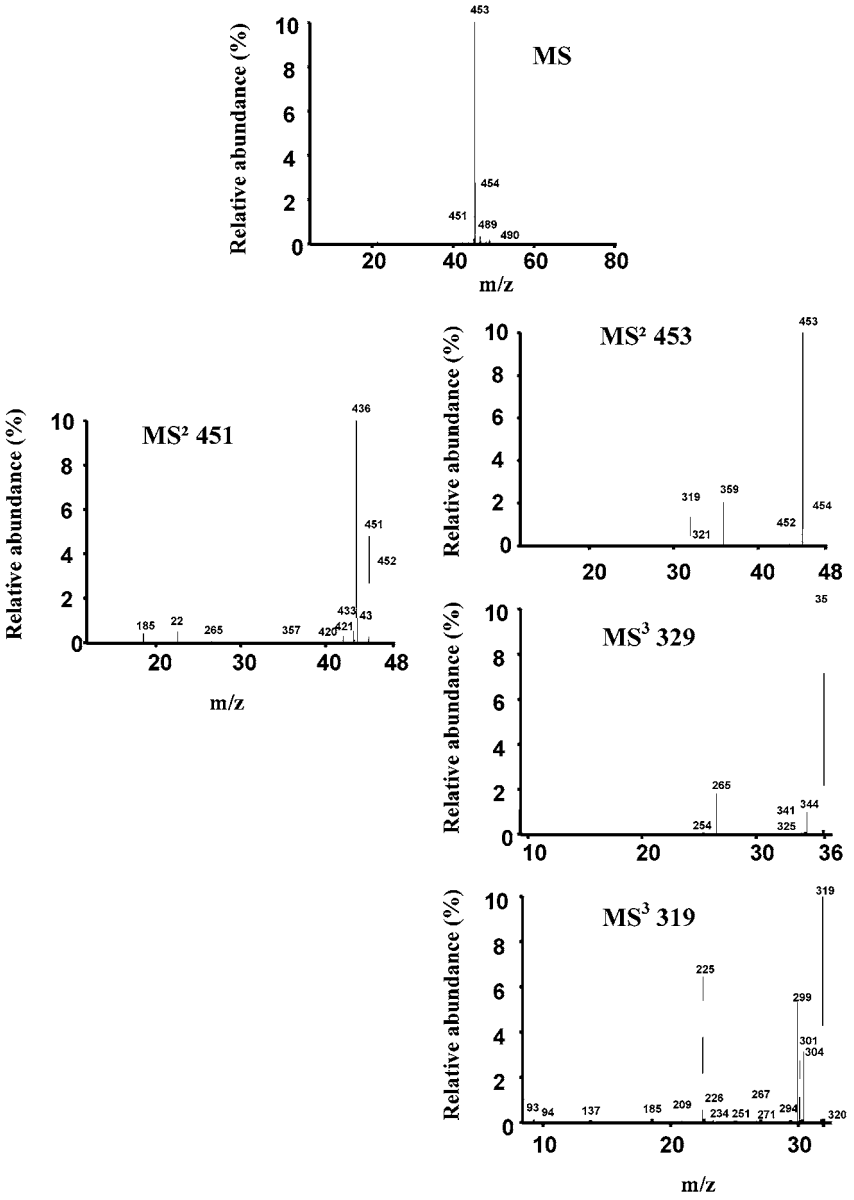


Figure 3. LC/ESI-MS^2 analysis of BPA dimers (peaks 8 and 9).

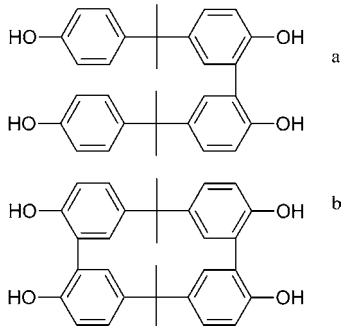


Figure 4. Proposed molecular structures for BPA dimers: (**a**) linear dimer isomer (ortho/ortho isomer; other possible structures; meta/ortho or meta/meta); (**b**) cyclic dimer (ortho/ortho isomer; other possible structures; meta/ortho or meta/meta).

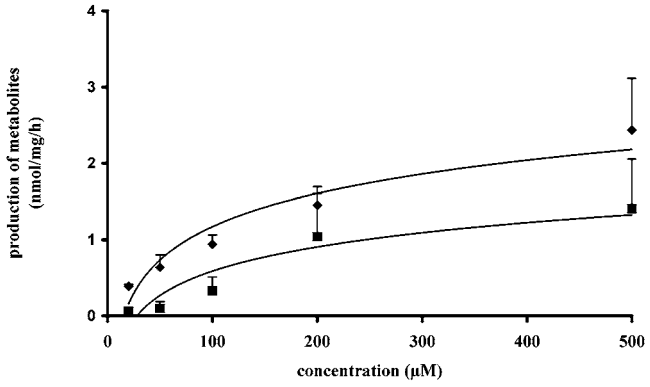


Figure 5. Microsomal production of isopropyl-hydroxyphenol (◆) and 5-hydroxylbisphenol A (■) as a function of substrate concentration. Data are expressed as mean ± SD ($n = 6$).

thionyl-phenol (R_T 10.5 min), glutathionyl 4-isopropylphenol (R_T 12.9 min), glutathione bisphenol A conjugate (R_T 23.4) and the two dimers (R_T 45 and 46.30 min, respectively). The structure of BPA glucuronide (R_T 24.20 min) was confirmed by cochromatography with the authentic standard synthesized in vitro and by mass spectrometry. Whatever the concentration of BPA incubated, the detected amount of BPA-glucuronide was always lower than the detected amount of glutathione BPA conjugate (R_T 23.4) (**Figure 8**).

NADPH was required for glutathione conjugate synthesis and when 100 μM glutathione reduced was added to the incubation

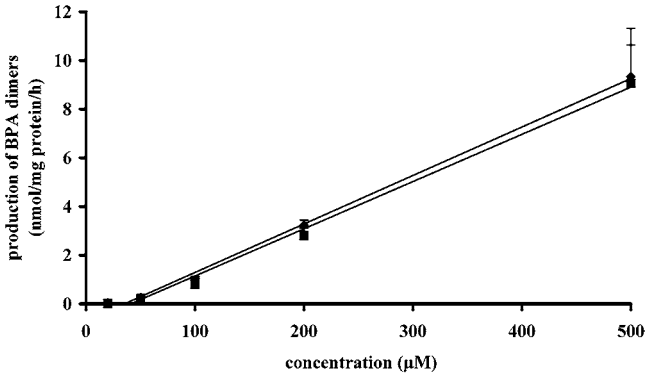


Figure 6. Microsomal production of BPA dimers [(◆) peak 8; (■) peak 9] as a function of substrate concentration. Data are expressed as mean ± SD ($n = 6$).

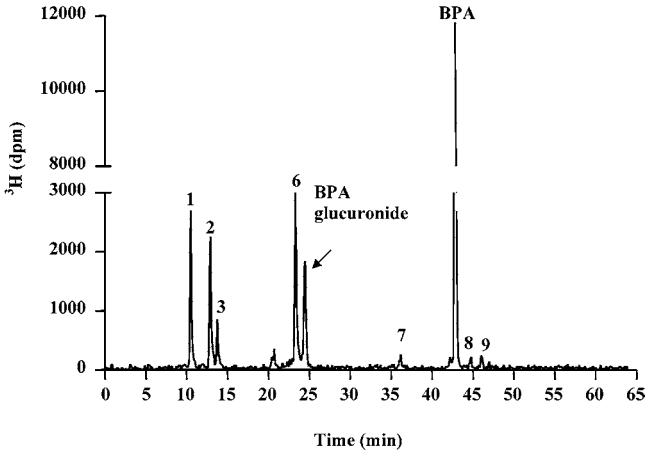


Figure 7. Representative HPLC-radiochromatogram obtained after incubation of BPA (100 μM, 2 h, 37 °C) with CD1 mice liver S9 fraction.

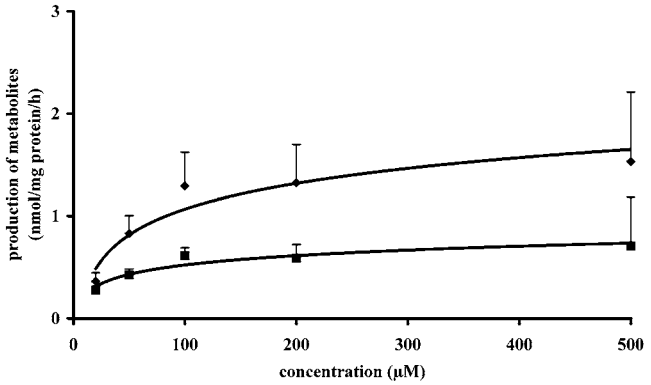


Figure 8. S9 fraction production of glutathionyl BPA (◆) and BPA glucuronide (■) as a function of substrate concentration. Data are expressed as mean ± SD ($n = 6$).

medium, production of glutathione conjugates increased whereas production of dimers did not.

When PAPS was added to S9 fraction incubations, BPA sulfate was detected at an R_T of 27.50 min (data not shown), but this metabolite was never observed in vitro without PAPS.

DISCUSSION

BPA possesses an estrogenic activity (*15*, *16*), can induce oxidative stress (*10*, *11*), and produces nucleotide adducts (*12*, *13*). However, the molecular mechanisms of BPA toxicity are not fully understood. To better understand these toxicological events and the possible associated health hazards for humans due to chronic exposure to BPA, it is necessary to investigate the metabolic fate of this xenobiotic, as well as the conditions in which human exposure occurs. To explore BPA metabolism, we used two complementary in vitro systems: CD1 mice liver microsomes and S9 fractions.

No major differences in the metabolism of BPA were observed between females and males, either using microsomes or S9 fractions. These observations are consistent with the results obtained via in vitro studies in humans and reported by Elsby (*26*), as well as with in vivo studies carried out in humans (*21*). However, gender-related differences in the metabolic pathways of BPA have previously been demonstrated in cynomolgus monkeys (*20*).

We isolated and characterized nine metabolites, including two metabolites exhibiting, in our system, a lower polarity than BPA. Some of them, BPA glucuronide and 5-hydroxybisphenol A, had already been described in different in vitro studies, but many metabolites are characterized here for the first time in mammals: glutathione conjugates, metabolites produced by the cleavage of BPA, and BPA dimers.

Glutathione BPA conjugates were not produced without NADPH, even when glutathione was added to microsomal and S9 incubations, suggesting the formation of a reactive intermediate. Two hypotheses explaining the formation of glutathione conjugates can be proposed. BPA could first be oxidized into an arene epoxide and subsequently conjugated to glutathione, this reaction being catalyzed by a glutathione S transferase (GST). Classically, when glutathione adds to an arene epoxide, the net result is a dihydroxyl-*S*-glutathionyl metabolite. However, in our study, we characterized a glutathionyl BPA that could be produced by the rearomatization of the ring and the loss of H_2O (*30*, *31*). The glutathione conjugate metabolites are quantitatively minor ones when BPA is incubated with CD1 liver microsomes, each peak accounting for less than 2% of the total radioactivity in 20 μM BPA incubations. However, they are quantitatively major metabolites when BPA is incubated with CD1 liver S9 fractions, each representing 15−20% of the total amount of radioactivity detected. These microsomal glutathione conjugate metabolites may be produced either by microsomal GST (mGST), with poor efficiency, or by the cytosolic GST (cGST) present in microsomal fractions (*32*). Microsomal and S9 fractions contain different GSTs and mGST that are distinct from cGST, are present in small amounts and possess specific substrates (*33*, *34*).

The second mechanism that can be hypothesized to explain the formation of these glutathione conjugates is a first-step CYP-mediated oxidation of BPA into 5-hydroxybisphenol A (catechol BPA, identified in our incubations), this intermediate possibly being converted into bisphenol-*o*-quinone. *o*-Quinones are electrophile-reactive species that can covalently bind to nucleophilic sites (*35*) and glutathione, a nucleophile compound, may react directly with bisphenol-o-quinone to produce glutathione BPA conjugates. This hypothesis is supported by the fact that S9 fractions, which contain more glutathione than microsomes, produced more glutathione conjugates, and that BPA conjugate production increased when glutathione was added to the incubation medium (data not shown). In addition, Atkinson and Roy (*12*, *13*) have already provided evidence that bisphenol-*o*-quinone can be formed in vitro and that this reactive intermediate is able to covalently bind to DNA.

Metabolites resulting from the cleavage of BPA: isopropyl-hydroxyphenol, glutathionyl-phenol, and glutathionyl 4-isopropylphenol were produced in vitro but the biochemical pathway

explaining the formation of these metabolites is not fully understood. No other studies have, to date, reported the occurrence of these metabolites in mammals. However a Gram-negative aerobic bacterium (strain MV1) has been shown to degrade BPA into many metabolites including 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, and 4-hydroxyacetophenone (*36*). None of these compounds was characterized in our study. Hirano (*37*) showed that a manganese peroxidase produced by a fungus (*Pleurotus ostreatus*) can degrade BPA into phenol, isopropyl phenol, and isopropenyl phenol, and that H_2O_2 is necessary to cleave the C−C bond between the central carbon and one of the aromatic rings of the molecule. Our experiments indicate that the cleavage of BPA only occurs in the presence of NADPH. In addition, if H_2O_2 and soybean peroxidase are added to either microsomal or S9 incubations, an increase in the production of metabolites resulting from the cleavage of BPA is observed, while 5-hydroxybisphenol A (catechol BPA) is no longer detected (data not shown). Consequently, BPA cleavage may be achieved by a peroxidase, after a first-step CYP oxidation of BPA into catechol BPA.

The characterized BPA dimers, which exhibited lower polarity than BPA in our HPLC system, were produced in higher quantities with liver microsomes than when incubated with S9 fractions. An LC-MS system was developed in order to further study these metabolites and led us to the tentative characterization of aliphatic dimers (two isomers) and of a cyclic dimer. The increase in the amount of dimers was proportional to the concentration of BPA incubated and even at 500 μM BPA, saturation was not reached. In addition, these dimers were not formed in the absence of NADPH. We hypothesize that BPA dimers formation may be a two-step metabolic pathway initiated by the enzymatic oxidation of BPA into an unidentified reactive intermediate, followed by a nonenzymatic reaction between the intermediate reactive compound and unchanged BPA. A polyphenoloxidase may be implicated in dimer production, as suggested by a study by Guyot (*38*), demonstrating that polyphenoloxidase catalyses the dimerization of catechin.

Snyder (*19*), studying the metabolism of BPA by HepG2 cells, observed an HPLC peak corresponding to a compound with a low polarity, which however could not be characterized. Metabolites exhibiting a similar polarity were described by Yoshihara (*16*), when incubating BPA with rat liver S9 fractions, but not with microsomal or cytosolic incubations alone. These metabolites were not characterized, but Yoshihara et al. (*26*) found that one of them exhibited higher estrogenicity than BPA. Several BPA metabolites have already been tested to characterize their estrogenic potency: BPA glucuronide was shown to be far less estrogenic than BPA; 5-hydroxy BPA was also demonstrated to be a weak estrogenomimetic compound (*25*, *26*), as was BPA sulfate (*39*). None of these metabolites is the estrogenic compound isolated by Yoshihara (*16*), which, on the basis of its weak polarity, might have been one of the dimers we characterized. However, Yoshihara (*16*) did not observe the formation of this metabolite in BPA incubations with microsomes, while, in our study, microsomes produced more dimers than S9 fractions. Based on the mass spectral data provided by these authors, its appears that the nonpolar metabolite they isolated was possibly a dimer of propenylphenol. Thus, the in vitro metabolization of BPA may lead to the formation of both BPA dimers (metabolites 8 and 9) and BPA metabolites (isopropylphenol and/or isopropyl-hydroxyphenol) dimers. In this study, the amount of BPA dimers we were able to isolate was too small to test their estrogenic activity. Additional studies will be necessary to clarify this point.

We identified several new BPA metabolites: glutathione BPA conjugates, metabolites resulting from BPA cleavage, and dimers. A reactive intermediate is necessary to produce these different metabolites. There is indirect evidence suggesting that the bisphenol A-ortho-quinone, produced from 5-hydroxy bisphenol A (catechol), may be the reactive intermediate. Additional experiments are necessary to explore the mechanisms involved in these pathways and to identify the enzymes that play a role in the formation of these intermediates.

ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; BPA, bisphenol A; CYP, cytochrome P 450; ESI, electrospray ionization; GST, glutathione S transferase; HPLC, high-performance liquid chromatography; LC-MS, combined high-performance liquid chromatography/mass spectrometry; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate reduced; PAPS, 3′-phosphoadenosine-5-phosphosulfate; R_T, retention time.

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