

Comparison of Conjugative Activity, Conversion of Bisphenol A to Bisphenol A Glucuronide, in Fetal and Mature Male Rat

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We showed previously that orally administered bisphenol A (BPA) easily crosses the placental barrier and enters the fetus. However, BPA glucuronide transport and metabolism in the fetus was not studied. We examined the transport of orally administered BPA and BPA glucuronide into mature rat testis and fetus of pregnant rats. After administration of an oral dose of 10 mg BPA per kg body weight to pregnant female rats, BPA glucuronide in the fetus was not detected. BPA glucuronide does not easily pass through the placental barrier. One hour after oral administration of 10 mg BPA per kg body weight to mature male rats, approximately 90% of the BPA was present as BPA glucuronide in both blood plasma and testis. Although the concentration of free BPA in blood plasma decreased gradually, free BPA in the testis had increased slightly 8 h after administration. Eight hours after oral administration of BPA, BPA glucuronide gradually decreased in rat testis. In contrast, following oral BPA administration, blood plasma BPA glucuronide decreased to 55% of the maximum observed concentration after 3 h, but then increased to 100% of the maximum observed concentration after 8 h. These results suggest that BPA easily passes through the testicular barrier, is converted by UDP-glucuronosyltransferase to BPA glucuronide, and gradually breaks down to BPA by β -glucuronidase.

Key words — bisphenol A, placental barrier, testicular barrier, UDP-glucuronosyltransferase, fetus, testis

INTRODUCTION

Bisphenol A (BPA; 4,4'-isopropylidenediphenol) is widely used by the chemical industry in the manufacture of polycarbonate and epoxy resins and as a stabilizing material or antioxidant for many types of plastics such as polyvinyl chloride.¹⁾ Polycarbonates are commonly used to line food cans, and BPA can leach from lacquer-coated cans^{2–4)} and baby feeding bottles.⁵⁾ BPA stimulates α and β estrogen receptors (ERs) at concentrations of 100–1000 nM^{6,7)} and induces proliferation of MCF-7 human breast cancer cells.^{8,9)} Vom Saal *et al.* reported that sperm production was 20% less in male offspring of female mice fed 20 ng BPA per g body weight during gestation days 11–17 than in male offspring of female mice fed a control diet during the same gestation

period.^{10,11)}

The placenta is an effective barrier against proteins or foreign bodies that might be harmful to the fetus. The placenta likely also protects the developing embryo against hormones in the maternal blood that might adversely affect fetal development.^{12,13)} The placental barrier is impervious to most sex hormones including estrogen. However we found BPA in maternal blood and the fetus immediately after oral administration to the pregnant rats.¹⁴⁾ Therefore, we are concerned as to whether BPA enters testicular tissue during the developmental stages of the fetus and affects sperm production or viability of adult male animals.

Generally, UDP-glucuronosyltransferase (UGT) is present mainly in the liver but recent studies revealed that it is present at substantial levels in the testis of rat,¹⁵⁾ monkey¹⁶⁾ and human,¹⁷⁾ and in human placenta.¹⁷⁾ However, it is not known whether UGT activity is present in fetus. β -Glucuronidase, an enzyme that catalyzes the hydrolysis of β -glucuronic acid terminally linked with BPA and other carbohydrates, is present in lysosomes of Sertoli cells,

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germ cells, and interstitial cells from rat testis¹⁸⁾ and lysosomes of germ cells and spermatozoa from mouse testis.¹⁹⁾ Thus, the localization of UGT and β -glucuronidase suggests that BPA may also exist as both a free form and a β -glucuronic acid linked form in these cell types. Therefore we examined the transport of orally administered BPA to the testis in mature male rats and to the fetus of pregnant rats and compared the amounts of intact BPA and BPA glucuronide. (This research was presented in part at the Meeting for the Japan Society of Endocrine Disrupters Research held at Kyoto in December 1999).

MATERIALS AND METHODS

Reagents — Solvents and reagents were analytical grade and were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The purity of BPA was over 99.0%. D₁₀-Fluoranthene, greater than 98.0% pure, was purchased from C/D/N Isotopes (Quebec, Canada). β -Glucuronidase enzyme from *Helix pomatia* type HP-2 (containing 100000 units/ml β -glucuronidase and substantial arylsulfatase activity) was purchased from Sigma Chemical Co., Inc. (St. Louis, MO, U.S.A.). Water was distilled and then purified with a Milli-Q™ Type I Reagent Water System. Solid phase extraction (SPE) of BPA was carried out with GL-Pak PLS-2 cartridges (500 mg/6 ml) from GL Sciences, Inc. (Tokyo, Japan). Prior to use, the cartridges were washed with 10 ml of methanol, 10 ml of water, and then 10 ml of 0.01 N HCl.

Animals — Pregnant Wistar rats (gestation days 12–14) and mature male rats (9 weeks old) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). The animals were kept in a room maintained at 23°C and 50% relative humidity. The animals were housed two per cage, fed rat chow (CE-2) and provided tap water *ad libitum*. One day before BPA administration, the rats were fasted and provided only tap water. We gave pregnant rats an oral dose of 10 mg BPA per kg body weight on gestation day 19. One hour after BPA administration, fetuses were removed from the pregnant rats. One, three and eight hours after oral administration of 10 mg BPA per kg body weight to 10-week-old male rats, blood was drawn, plasma was prepared, and testes were removed.

Extraction and Acetylation of BPA — Blood plasma of mature male rats: Blood plasma (1.5–2 ml) of mature male rats was added to 5 ml of 1.1 M ac-

etate buffer (pH 5.2). The samples were treated without or with 50 ml of β -glucuronidase at 55°C for 2 h on a water bath, and later cooled to room temperature.²⁰⁾ Samples were then passed through SPE cartridges, and the organic compounds remaining on the disk were eluted with 10 ml of methanol. The eluates were evaporated, and 1 ml of pyridine and 1 ml of acetic anhydride were added to the residue and incubated for 20 min at 70°C.^{21,22)} Water (3 ml) and hydrochloric acid (1 ml) were added. BPA was extracted twice with 3 ml of toluene, and the toluene layer was evaporated to near dryness.

Preparation of tissue extracts: Whole organs from rat fetuses (2–3 g) and testes (1.5–2 g) from mature male rats were homogenized in 5 ml of 1.1 M acetate buffer (pH 5.2). The homogenate was treated without or with 150 ml of β -glucuronidase at 55°C for 2 h on a water bath and then cooled to room temperature. The homogenate was centrifuged, and the supernatant was transferred to a glass tube. The residue was homogenized with acidic methanol (1 volume 0.1 N HCl, 9 volumes methanol). Each homogenate was transferred to a glass tube and added 0.01 N HCl up to 150 ml. Samples were then applied to SPE cartridges and subjected to the same procedures described above for extraction and acetylation of BPA from blood plasma. A flow chart for this method is shown in Fig. 1.

GL-Pak PLS-2 cartridges (polystyrene divinyl benzene polymer) were used instead of either octadecyl (C18) or cyclohexyl (CH) column for SPE because they provide higher yields of BPA, are more stable, and therefore, do not contaminate the samples with material that leaches off of the resin under acidic conditions. Since low recovery of BPA was observed when the columns were allowed to dry completely, residual water was removed by placing the columns under vacuum for 30 s. BPA was reacted with acetic anhydride in pyridine since water does not interfere with this reaction.

Determination of BPA by Gas Chromatography/Mass Spectrometry (GC/MS) — Qualitative and quantitative analyses was performed with an HP model 5890 II gas chromatograph (Yokogawa-Hewlett-Packard Ltd., Tokyo, Japan) equipped with a JEOL Automass 50 mass spectrometer (JEOL Ltd., Tokyo, Japan). The analytical column was a CP SIL 19CB, 25 m × 0.25 mm i.d., and 0.20 μ m film thickness (Chrompack, The Netherlands).

Immediately prior to GC/MS analysis, 100 μ l of d₁₀-fluoranthene, a deuterated internal standard (i.s.) for quantitation, was added to each extract.²¹⁾

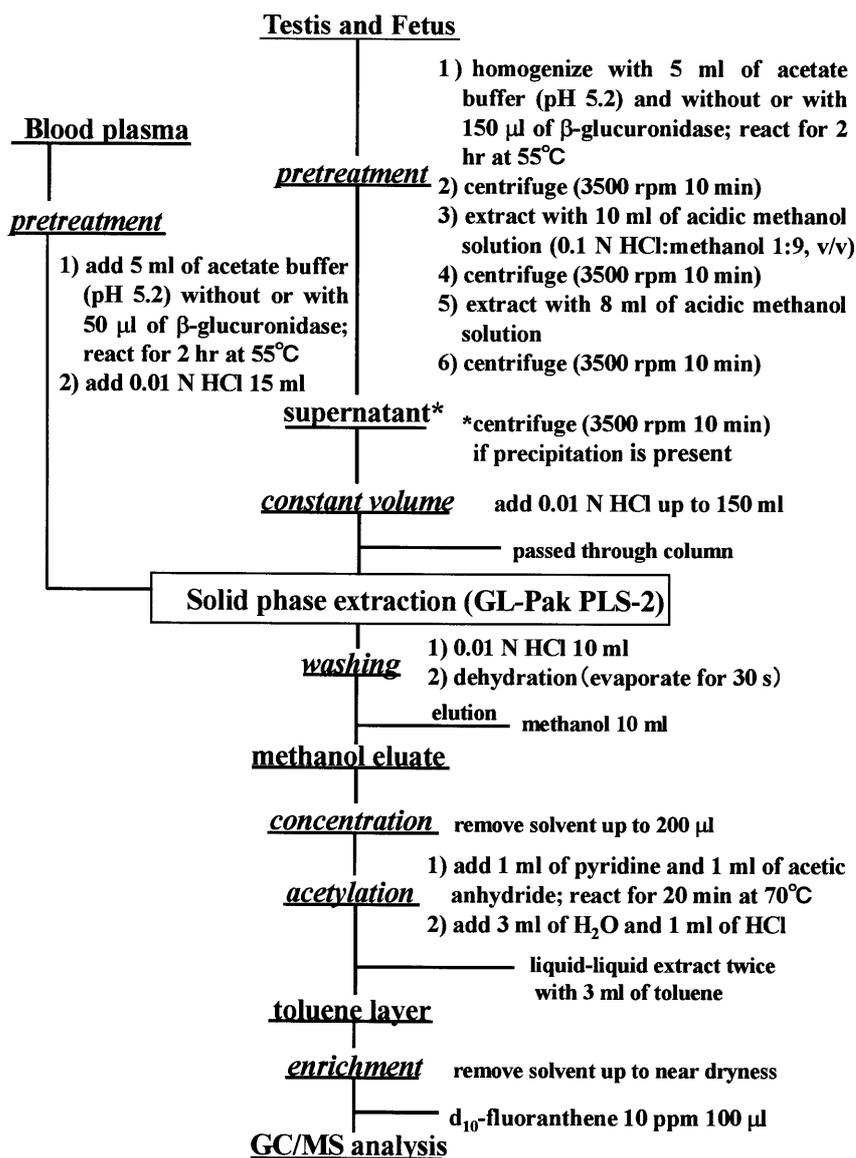


Fig. 1. Flow Chart of the Method for Determining BPA Concentrations

Splitless injection of sample (1 μ l) into the GC/MS was performed at 250°C. The carrier gas was helium, and the flow rate was 1.0 μ l/min. The oven temperature was initially 80°C for 2 min and was then programmed to increase to 200°C at 20°C/min and held for 2 min. It was then increased further to 270°C at 5°C/min (for blood plasma and testes) or 3°C/min (for rat fetuses), and held for 10 min. The electron impact ionization conditions were as follows: ion energy, 70 eV; ion source temperature, 200°C; mass range, m/z 50–500 full scan for qualitative analysis.

Acetylated BPA was measured by GC/MS in the selected ion monitoring mode (GC/MS-SIM) using d_{10} -fluoranthene. The m/z of the most stable and

abundant ions were 119, 213, 270, and 312 for acetylated BPA and 212, and 213 for d_{10} -fluoranthene. Ion m/z 213 for acetylated-BPA and d_{10} -fluoranthene were used for the quantification of BPA.

RESULTS AND DISCUSSION

To determine recovery of BPA, we added a standard solution of BPA to blood plasma, fetus, and testis samples, and then performed the entire analytical procedure. The mean recovery was approximately 90%, and the standard deviation was less than 8% for each biological sample.

In an earlier study, we reported that BPA was

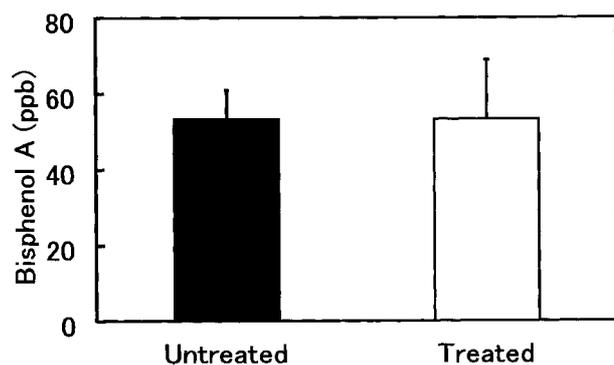


Fig. 2. Concentrations of BPA in Untreated and β -Glucuronidase-Treated Extracts from Fetuses of Pregnant Rats after Oral Administration

One hour after exposure, the concentration of BPA in day 19 fetuses were measured as described in the Materials and Methods. \square , Fetal extracts treated with β -glucuronidase; \blacksquare , Untreated fetal extracts. Values are mean \pm S.D. of 4 separate experiments.

present in fetuses of pregnant rats receiving a single oral dose of 10 mg BPA per kg body weight.¹⁴⁾ However, we did not examine whether BPA glucuronide, which can be formed from BPA by the action of UGT, also passes through the placental barrier to the fetuses. Pregnant rats were treated with a single oral dose of 10 mg BPA per kg body weight, and the quantity of BPA in fetuses was determined after treating extracts without or with β -glucuronidase (Fig. 2). No significant differences in BPA concentration were detected between untreated and β -glucuronidase-treated fetal extracts. There are several explanations for this result. For example, it is possible that BPA can not pass through the placental barrier. Also, it is not likely that BPA is glucuronidated in rat fetus, suggesting that UGT is not present or does not have detectable activity.

Total ion chromatograms for extracts from blood plasma and testis displaying peaks for acetylated BPA ($m/z = 213$) appearing at 19.54 min (Fig. 3) were used to determine the detection limit with a signal-to-noise ratio of 3. The detection limit of acetylated BPA was approximately 30 pg when 1 μ l of the extract was injected onto the GC column. Identification of the signal component at 19.54 min was performed by comparison of the corresponding mass spectrum and retention time of a standard acetylated BPA solution. Thus, we confirmed that BPA from blood plasma and testis could be detected and quantitated using this extraction protocol and GC/MS.

One hour after administration of an oral dose of 10 mg BPA per kg body weight to mature male rats, approximately 90% of the BPA was present as BPA

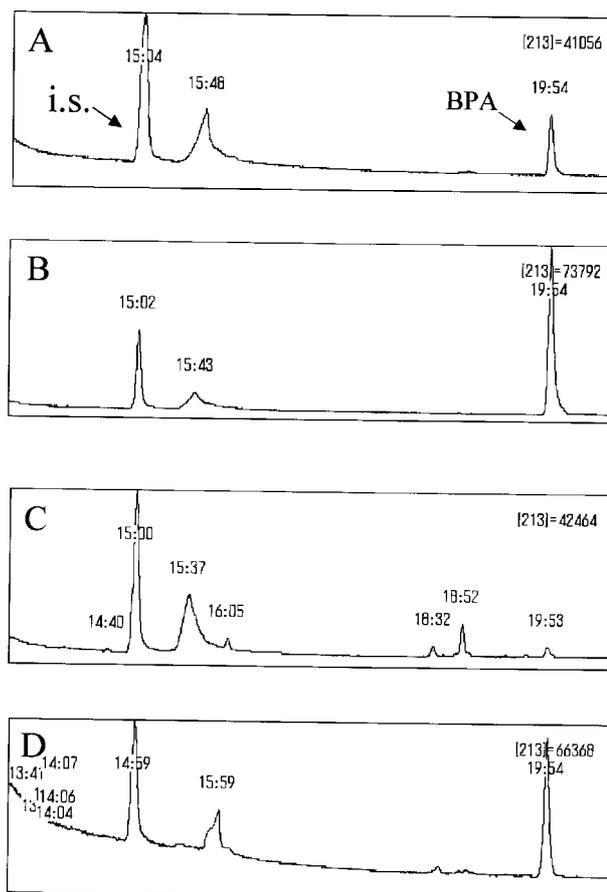


Fig. 3. Total Ion Chromatograms of Extracts of Blood Plasma and Testis

One hour after oral administration of 10 mg BPA per kg body weight, plasma and testis extracts were prepared from mature male rats as described in the Materials and Methods. (A) Untreated blood plasma. (B) Blood plasma treated with β -glucuronidase. (C) Untreated testis extracts. (D) Testis extracts treated with β -glucuronidase.

glucuronide in both blood plasma and testes (Figs. 4 and 5). Intact BPA steadily decreased in blood plasma and increased slightly in testis 8 h after administration. In contrast to the gradual decrease of BPA glucuronide observed in testis, the level of BPA glucuronide in blood plasma measured as a percentage of the maximum concentration detected following oral BPA administration first decreased to 55% at 3 h and then increased to 100% at 8 h. Kurebayashi *et al.* reported that the concentration of BPA glucuronide in rat blood increased 4 h after oral BPA administration as measured by the biliary excretion of BPA glucuronide through the enterohepatic circulation (Kurebayashi H. *et al.*, abstract from XXVth symposium on Toxicology and Environmental Health, P-75, 1999). We also observed an increase of BPA glucuronide concentration in blood of mature rats after oral BPA administration.

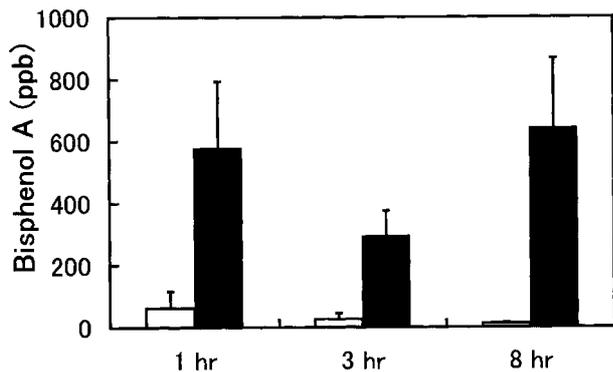


Fig. 4. Concentrations of BPA and BPA Glucuronide in Blood Plasma from Mature Male Rats after Oral Administration

One hour, 3 h, and 8 h following oral administration of 10 mg BPA per kg body weight to 10-week-old male rats, the concentration of BPA and BPA glucuronide in blood plasma were determined as described in the Materials and Methods. □, Free BPA; ■, BPA glucuronide. Values are mean ± S.D. of 4 separate experiments.

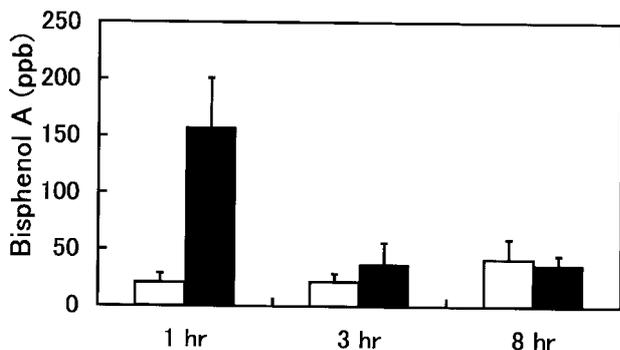


Fig. 5. Concentrations of BPA and BPA Glucuronide in Testis from Mature Male Rats after Oral Administration

One hour, 3 h, and 8 h following oral administration of 10 mg BPA per kg body weight to 10-week-old male rats, the concentration of BPA and BPA glucuronide in testes were determined as described in the Materials and Methods. □, Free BPA; ■, BPA glucuronide. Values are mean ± S.D. of 4 separate experiments.

In general, the membrane permeability of lipid-soluble BPA [$\log P_{ow}$ (partition coefficient for octanol/water) = 3.32] is high while that of water-soluble BPA glucuronide is low. Based on the concentration of BPA glucuronide in rat testis (40 ppb) and blood plasma (600 ppb) 8 h after oral BPA administration, BPA glucuronide did not seem to pass through the testicular barrier. Since 90% of the total BPA in mature rat testis was in the BPA glucuronide form 1 h after oral BPA administration, it seems likely that free BPA crossed the testicular barrier and was subsequently converted to BPA glucuronide by the action of UGT. Free BPA may gradually accumulate in mature rat testis following oral adminis-

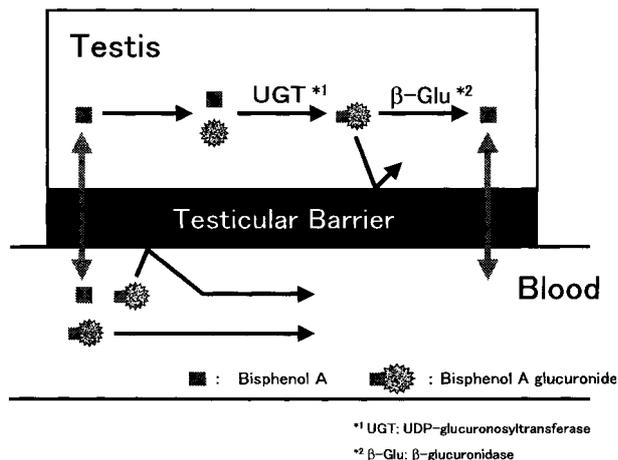


Fig. 6. Passage of BPA and BPA Glucuronide through the Testicular Barrier

tration because β-glucuronidase would continue to catalyze the breakdown of BPA glucuronide, and conversion of BPA to BPA glucuronide by UGT would slow possibly due to exhaustion of UDP cosubstrate. Thus, this scheme (Fig. 6) would explain why more BPA existed in the free form than in glucuronic acid form in mature rat testis 8 h after oral administration.

In mature rat liver microsomes, the UGT isoform UGT2B1 catalyzes glucuronidation of BPA.¹⁵⁾ Although the transitional ratio of BPA is low, we cannot predict the effect of BPA on rat fetuses. Even though BPA significantly reduces sperm production in male offspring of female mice fed BPA during pregnancy,^{10,11)} we believe that BPA has little toxic effect on rat testis because mature rats display normal UGT activity in testis as well as other organs. However, BPA may be toxic to the fetus since UGT can not be detected during development. The effect of BPA on the male reproductive system remains controversial. Moreover, little is known about the pharmacokinetics and toxicity of BPA in other organ systems such as the brain. Therefore, further study of BPA toxicity is necessary to insure public health and safety.

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