ELSEVIER

Life Sciences 71 (2002) 887–898

Life Sciences

www.elsevier.com/locate/lifescie

High glucuronidation activity of environmental estrogens in the carp (*Cyprinus carpino)* intestine

Hiroshi Yokota *, Naoki Miyashita, Akira Yuasa

Department of Veterinary Biochemistry, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

Received 7 June 2001; accepted 25 January 2002

Abstract

Many adverse effects on carp reproductive organs have been reported to be caused by exposure to environmental estrogens, such as nonylphenol and bisphenol A, which contaminate the aquatic environment. The glucuronidation activities of xenoestrogens (bisphenol A and diethylstilbestrol) and phytoestrogens (coumestrol, genistein and biochanin A), but not nonylphenol and octylphenol, were observed in microsomes prepared from carp organs. The highest levels of glucuronidation of environmental estrogens, for which the optimum temperature was 25–30 °C, were observed in the intestinal microsomes of 2-year-old carp. These activities in carp intestine increased developmentally, and the maximum levels corresponded to 5–10 % of that in rat liver microsomes. However, the glucuronidation of phytoestrogen by carp intestinal microsomes corresponded to that of rat liver microsomes. Only bisphenol A-glucuronide was excreted from the everted intestine, indicating that bisphenol A is metabolized in the carp intestine mainly as glucuronide. These results suggest that glucuronidation by carp intestine plays an important role for the detoxification of xenoestrogens and phytoestrogens, except for nonylphenol and octylphenol.

Keywords: Glucuronidation; Xenoestrogen; Phytoestrogen; Carp; Intestine; Hepatopancreas; Bisphenol A; Nonylphenol; Octylphenol

* Corresponding author. Tel.: +81-11-388-4743; fax: +81-11-387-5890.
E-mail address: h-yokota@rakuno.ac.jp. (H. Yokota).

0024-3205/02/$ - see front matter
PII: S0024-3205(02)01767-8

Introduction

Much evidence has recently been reported on the hormone-like effects of environmental chemicals in fish, wildlife and humans [1]. Bisphenol A, which is a monomer used in the manufacture of polycarbonate, and alkylphenols (nonylphenol and octylphenol), which are compounds widely used as plastic additives and for the manufacture of surfactants, have been reported to have estrogenic activities [2–4]. Significant amounts of these alkylphenols have been found in the aquatic environment, especially in sediments [5,6]. Alkylphenolic compounds can bioaccumulate in fish and potentially impair reproduction or development through estrogenic actions [7–10]. Feminized male fishes have been found near sewage outlets in several rivers in the U.K.; a mixture of chemicals including alkylphenols resulting from degradation of detergents during sewage treatment seemed to be the causal agent of this endocrine disruption [11]. Fishes such as carp may be sensitive to these polluted chemicals. The precise mechanisms of action of a number of these xenoestrogens are still not known.

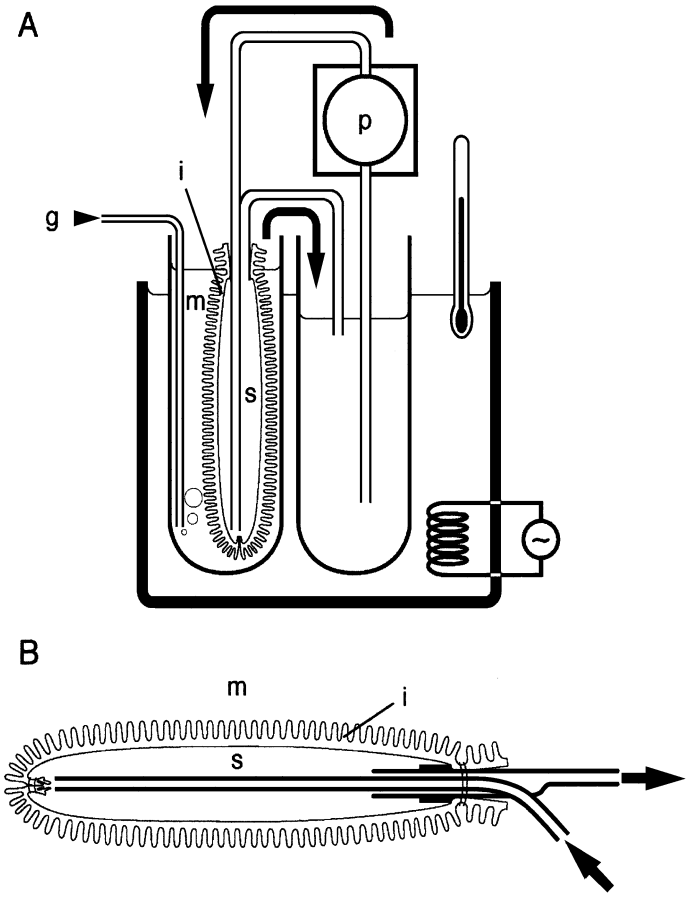

Fig. 1. Scheme of everted intestine system. Whole system (A) and the apparatus to which a segment of everted bowel is attached (B). The mucosal side (m) and serosal side (s) were filled with Krebs Ringer's buffer and the buffer was maintained at 37 °C in water bath. The mucosal buffer solution was gassed with 95% O_2–5% CO_2 (g). Arrows indicate the flow of the serosal buffer. Preparation and fixation of the aliquot of everted intestine (i) are explained in Materials and Methods.

Bisphenol A and nonylphenol are mainly glucuronidated in the liver and excreted into the bile of rats [12–14] and rainbow trout (*Oncorhynchus mykiss*) [15,16]. Recently, plasma xenoestrogen biomarkers such as viterogennin and *zona radiata* proteins were shown to be induced by intraperitoneal injections of bisphenol A and nonylphenol into juvenile salmon [17]. The metabolism of these chemicals in fish must be determined in order to elucidate the mechanisms of viterogennin induction and endocrine disruptions. It is basically important to know whether xenobiotic pollutants, such as bisphenol A and nonylphenol, are glucuronidated in the organs of fishes that have sustained adverse effects for estimation of toxicity of these chemicals.

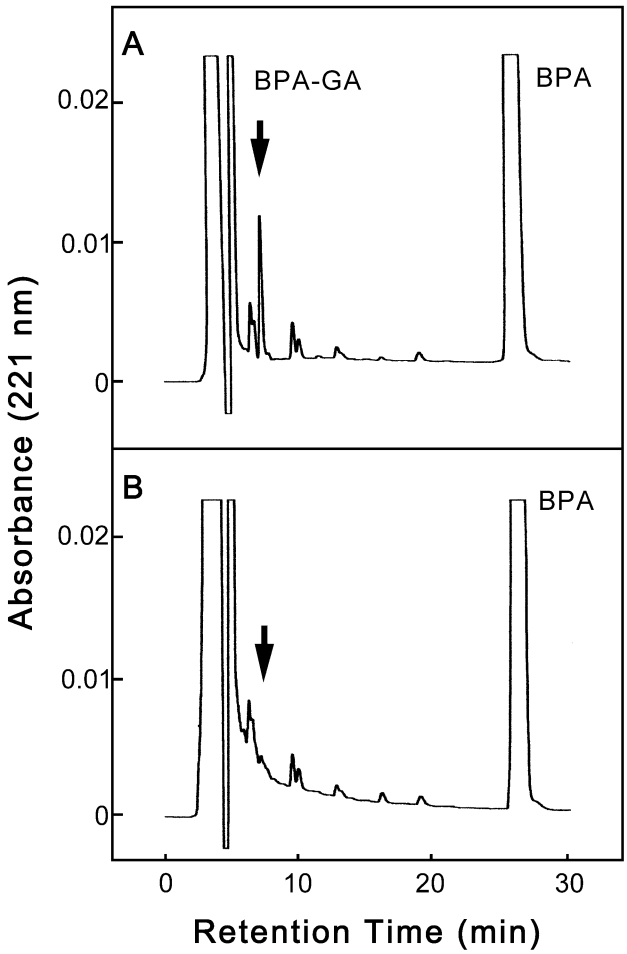

Fig. 2. HPLC analysis of the bisphenol A glucuronidation in carp hepatopancreas microsome. Chromatograms were generated from HPLC of the reaction products of bisphenol A (BPA) in carp hepatopancreas microsomes as described in *Materials and Methods*. Panel A shows a chromatogram of the reaction products of BPA in microsomes in the presence of UDP-glucuronic acid. Panel B is a chromatogram of β-glucuronidase treatment of the same products as those shown in chromatogram A. The peak eluted at 27 min in the chromatograms was estimated as unreacted BPA, and the peak eluted at 7 min (indicated by narrow) is abolished by the β-glucuronidase-treatment (panel B).

Materials and Methods

Materials

Cholic acid, purchased from Nissui Yakuhin Co., was further purified and converted to its sodium salt [18]. UDP-glucuronic acid was obtained from Nakarai Yakuhin Co. Bisphenol A, testosterone, estradiol, estradiol 17β-glucuronide and estradiol 3α-glucuronide were obtained from Sigma Chemicals. 1-

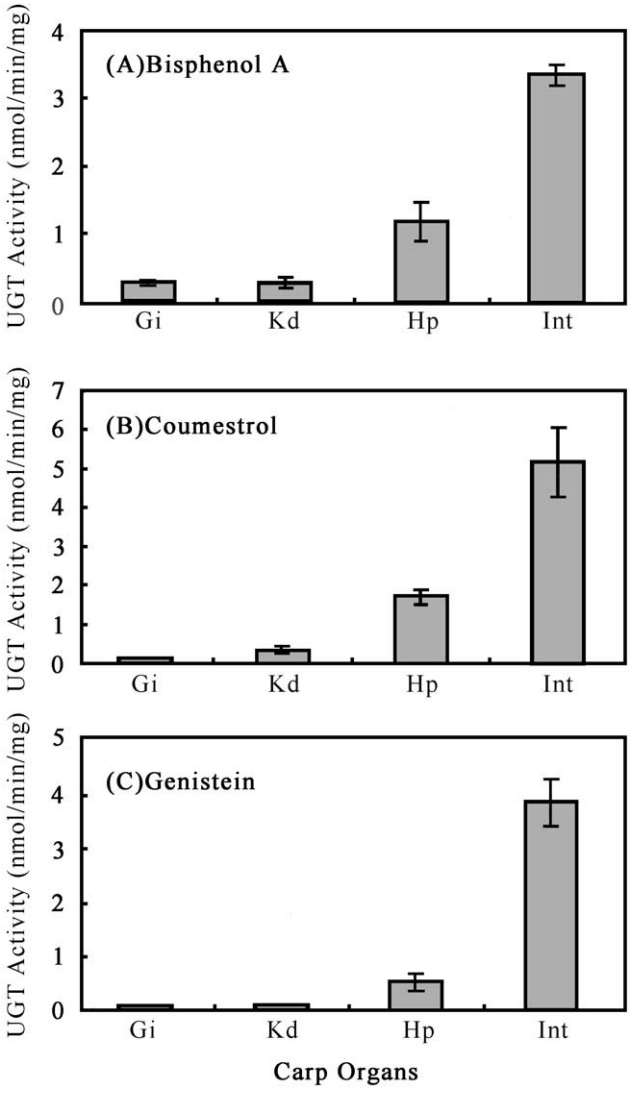

Fig. 3. Microsomal UDP-glucuronosyltransferase activities towards environmental estrogens by carp organs. Enzymatic activities toward bisphenol A (A), coumestrol (B) and genistein (C) were assayed in cholate-activated microsomes prepared from the carp intestine (In), hepatopancreas (Hp), kidney (Kd) and gill (Gi) at 25 °C as described in Materials and Methods. Results are means ± S. E. (error bar).

Naphthol (α-Naphthol) was purchased from Wako Chemical Co. (Osaka, Japan), and α-naphthyl β-D-glucuronide (1-naphthol-β-D-glucuronide) was purchased from Sigma Chemical Co. (St. Louis, MO., USA). HPLC-grade acetonitrile was obtained from Kanto Chemical Co. (Tokyo, Japan). Other reagents were of the highest grade available.

Preparation of microsomes from carp organs

The carp were dissected after exsanquination by decapitation in accordance with the Japanese Guideline for Experimental Animals, and the hepatopancreas, kidneys, intestines and gills were minced and homogenized with 4 vol. of 0.15 M KCl solution containing 1 mM EDTA. The homogenate was centrifuged for 30 min at 9,000 × *g*, and the supernatant fraction was centrifuged at 105,000 × *g* for 60 min to obtain microsomes. Rapid preparation of the carp microsomal fraction was important to prevent the loss of UGT activities. The microsomes were activated by the incubation with 0.01% sodium cholate for 30 min at 0 °C. The protein concentration was determined by the method of Lowry *et al*. [19] using bovine serum albumin as a standard.

Enzyme analysis and HPLC

UDP-glucuronosyltransferase activities toward various substrates were assayed in 200 μl of 50 mM Tris-HCl buffer (pH 7.4), 0.5 mM $MgCl_2$ containing 0.25 mM substrate and cholate-activated microsomes at 37 °C as previously described [12]. The resultant enzyme reaction products were filtered using a disposable disk filter (HPLC-DISK; Kanto Co., Tokyo, Japan) and analyzed by an HPLC system

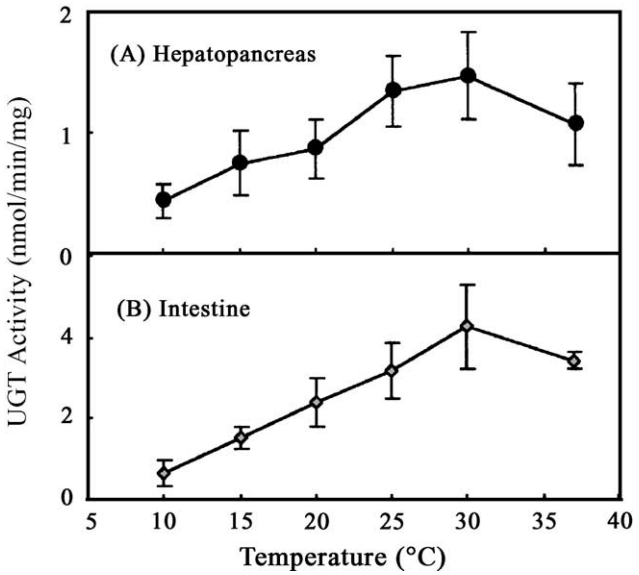

Fig. 4. Temperature dependency of microsomal UDP-glucuronosyltransferase activity toward bisphenol A in microsomes prepared from carp hepatopancreas and intestine. Enzymatic activity toward bisphenol A was assayed in cholate-activated microsomes prepared from the carp hepatopancreas (A) and intestine (B) at various temperatures for 10 minutes as described in Materials and Methods. Results are means ± S. E. (error bar).

consisting of a Tosoh TSKgel 80TM reversed phase column (7.8 mm × 30 cm). The filtered samples were injected and eluted with an acetonitrile/H_2O/acetic acid (35/65/0.1 v/v/v) solution. Substrate-glucuronides were estimated by decomposition with β-glucuronidase and quantified by the decrease in the substrate peak on a HPLC chromatogram. The HPLC peaks of estradiol 3α-glucuronide and estradiol 17β-glucuronide were determined by using respective authentic standards.

Setup of everted intestine system

Schemes of the everted intestine system were described previously [20]. A 16-cm-long polyethylene tube (1 mm in i. d., 1.66 mm in o.d.) was inserted to a length of 3 cm into an outer tube (2 mm in i.d., 3 mm in o.d., 3 cm in length), leaving 13 cm dangling free outside the apparatus. A silicon projection was added to the circumference near one end of the outer tube to fix the intestine. Krebs Ringer's buffer (Na^+ 135.0 mM, K^+ 5.0 mM, Ca^{2+} 2.5 mM, Mg^{2+} 1.2 mM, Cl^-122.4 mM, HCO^{3-}25.0 mM, glucose 10.0 mM) was aerated by 95% O_2 + 5% CO_2 gas, and the pH was adjusted to 7.4. Two test tubes were filled with 30 ml of Krebs Ringer's buffer and incubated in a water bath at 37 °C. The substrate (1-naphthol, final conc. = 0.05 mM) was added to the Krebs Ringer's buffer at the mucosal site. The mucosal site (m) was aerated with 95% O_2 + 5% CO_2 gas (g) to maintain viability of the tissue. Silicon tubes were used to make the connection between the pump and tissue in the step as described in Fig. 1.

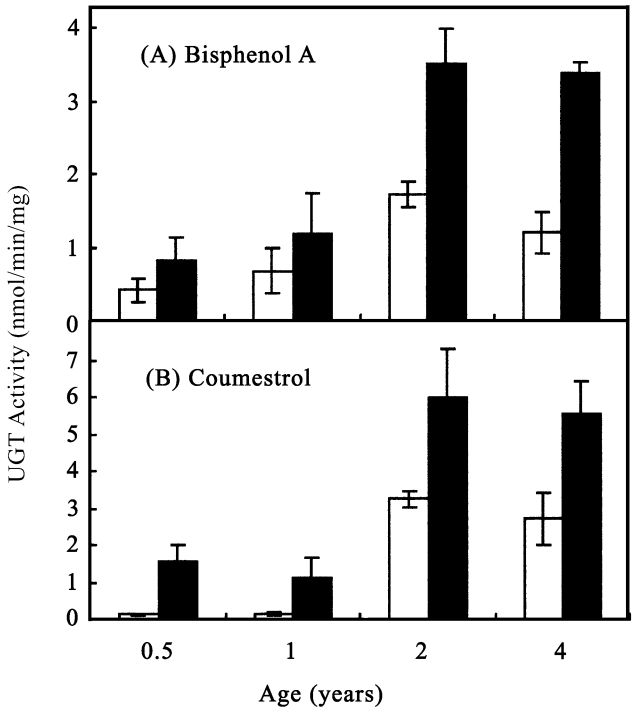

Fig. 5. Age dependency of microsomal UDP-glucuronosyltransferase activities toward environmental estrogens by microsomes prepared from carp hepatopancreas and intestine. The enzymatic activities towards bisphenol A (A) and coumestrol (B) were assayed in cholate-activated microsomes prepared from carp hepatopancreas (□) and intestine (■) at 25 °C as described in Materials and Methods. Results are means ± S. E. (error bar).

Preparation of everted intestine

The carp intestine was washed with cold Krebs Ringer's buffer. The washed intestine was immediately sectioned into two equal 15-cm lengths in cold Krebs Ringer's buffer. One end of each

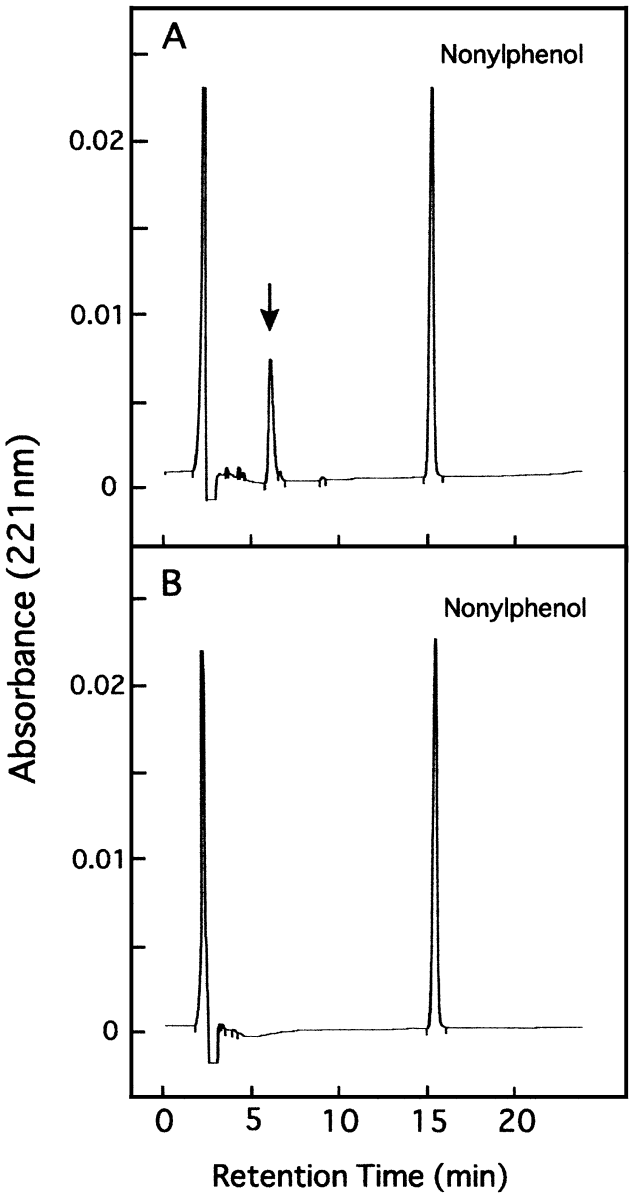

Fig. 6. HPLC analysis of nonylphenol glucuronidation in rat liver and carp intestinal microsomes. Chromatograms A and B were generated from the products of incubation reactions to nonylphenol with the rat liver microsomes (A) carp intestinal microsomes (B). Elution of unconjugated nonylphenol was confirmed as a gentle peak at 15.1 min. A major peak corresponding to nonylphenol glucuronide (arrowhead) in panel A was not detected in panel B.

of the sections was ligated and everted simultaneously with the insertion of the fixation equipment. The other end of the intestine was then ligated over the polyethylene tube. The everted segment was fixed in the mucosal buffer (m), and the serosal buffer was pumped (p) through the everted intestine at 5 ml per minute. Reaction products (0.5 ml of the mucosal and serosal buffer) were collected at 0, 10, 20 and 30 min after the addition of a substrate as shown in Fig. 1. Mucosal and serosal samples were filtered using a disposable disk filter (HLC-DISK$_{TM}$3; Kanto Co., Tokyo, Japan) and stored at $^{-}$80 °C until analysis. The samples were analyzed by an HPLC system.

Results

In this study, we found significant levels of UDP-glucuronosyltransferase activities toward environmental estrogens such as bisphenol A and some phytoestrogens, but not nonylphenol and octylphenol, in microsomes prepared from carp intestine. The results of HPLC analysis of the reaction products of bisphenol A obtained *in vitro* from carp hepatopancreas microsomes in the presence of UDP-glucuronic acid are shown in Fig. 2. Unconjugated bisphenol A in the absence of UDP-glucuronic acid was eluted at 27 min, which is the same elution time for standard bisphenol A (data not shown). The reaction products of bisphenol A in carp hepatopancreas microsomes contained a new peak, which disappeared after β-glucuronidase treatment and was subsequently comfirmed as bisphenol A-glucuronide (Fig. 2). The results indicate that carp hepatopancreas microsomes have glucuronidation activity for the environmental estrogen bisphenol A.

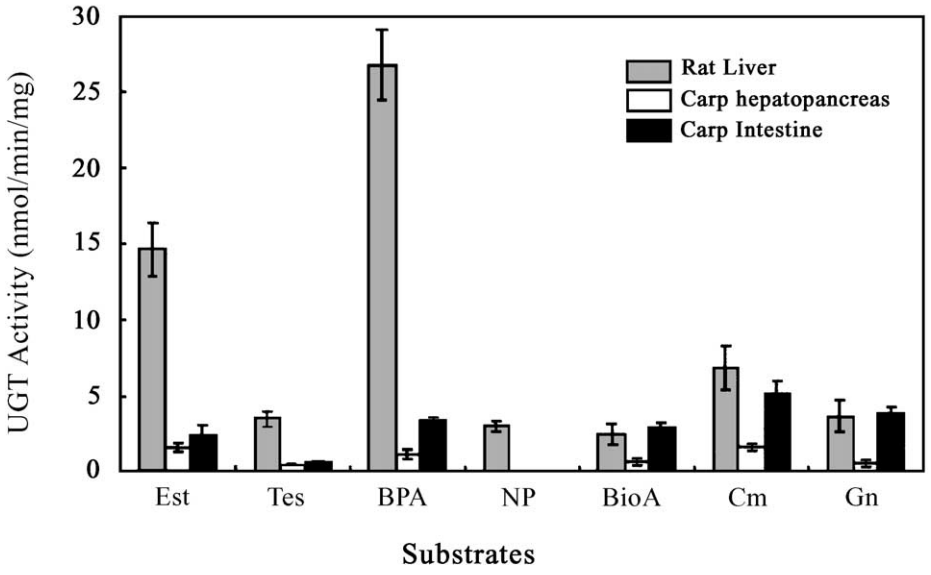

Fig. 7. Comparison of microsomal UDP-glucuronosyltransferase activities toward various substrates by microsomes prepared from rat liver and carp hepatopancreas and intestine. Enzymatic activities toward oestradiol (Est), testosterone (Tes), bisphenol A (BPA), nonylphenol (NP), biochanin A (BioA), coumestrol (Cm) and genistein (Gn) were assayed in cholate-activated microsomes prepared from the rat liver (▩) at 37 °C and from the carp hepatopancreas (□) and intestine (■) at 25 °C as described in Materials and Methods. Results are means ± S. E. (error bar).

In Fig. 3, xenoestrogens such as bisphenol A and also phytoestrogens were shown to be glucuronidated by microsomes prepared from carp, *Cyprinus carpino*, organs. The highest activities of UGT toward all substrates tested were obtained in microsomes from the intestinal mucosa, and only slight activities were detected in the gill. As shown in Fig. 4, the optimum temperature for the enzyme was lower (25–30 °C) than that for rat microsomes (35–45 °C). At temperatures higher than 30 °C, UGT activity was significantly decreased, suggesting that carp UGT protein is comparatively unstable at temperatures higher than 30 °C. All assays of carp enzyme activities were performed at 25 °C, which is a stable and active temperature for carp UGT. No significant loss of UGT activity in carp microsomes was observed at 25 °C for 1 hr (data not shown). Developmental changes in UGT activity toward bisphenol A and coumestrol are shown in Fig. 5. UGT activity increased slowly with carp development and reached its maximum level before the carp reached sexual maturity (2 years old) (Fig. 5). Nonylphenol glucuronide was detected after reaction with rat liver microsomes but not with carp intestine microsomes on HPLC chromatography as shown in Fig. 6. UGT activities toward various substrates in microsomes from adult carp organs and rat liver are shown in Fig. 7. The carp intestine showed higher (above 2-fold) activity than did carp hepatopancreas in the glucuronidation of bisphenol, biochanin A, coumestrol and genistein, but showed no activity for nonylphenol (Fig. 6) and otylphenol (data not shown). Carp intestinal UGT activities toward testosterone, estradiol and biphenol A corresponded to about 10% of that in rat liver microsomes, while even at 25 °C, the level of activity toward phytoestrogens was the same as that in

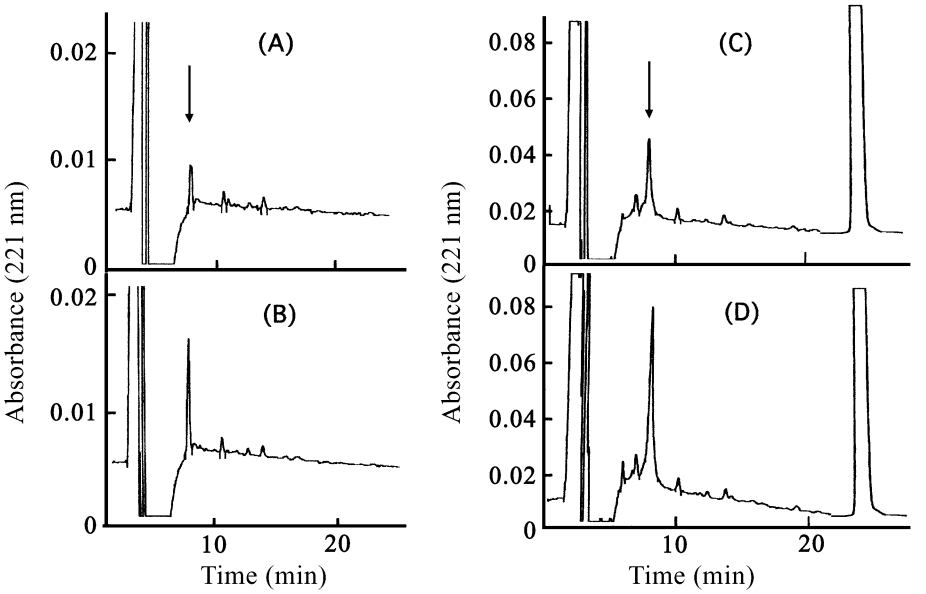

Fig. 8. HPLC analysis of bisphenol A-glucuronide secreted from the everted intestine of the carp. Bisphenol A was added to the mucosal side of the everted intestine, and the mucosal and serosal buffer solutions were assayed by HPLC as described in Materials and Methods. Chromatograms were generated from HPLC of the reaction products secreted into the serosal side (A and B) and the mucosal side (C and D) from the everted intestine after 10 min (A and C) and 30 min (B and D). Free bisphenol A was detected at 27 min only in the mucosal side and Arrows show the peaks of bisphenol A-glucuronide.

the rat liver (37 °C assay) (Fig. 7). To identify the main metabolite of bisphenol A in the carp intestine, reaction products excreted from the everted intestine were assayed by HPLC, and the results are shown in Fig. 8. A single peak of bisphenol A-glucuronide (panel A) was detected, which increased after reaction in the serosal and mucosal sides (panels B and D). Free bisphenol A was detected at 27 min only in the mucosal side, indicating that the main product of bisphenol A in the everted intestine was the glucuronide, and significant amounts of conjugated bisphenol A were excreted into the mucosal side of the intestine within 60 min.

Discussion

Xenoestrogens, bisphenol A [12,13] and nonylphenol [14], which are pollutants in the environment, were mainly metabolized to their glucuronide conjugates in the rat. Bisphenol A was shown to be glucuronidated at significant levels, corresponding to 10% of that in rat liver, in the carp intesitne, even at a lower assay temperature (25 °C). The expression of multiple UGT isoforms was reported in the liver of the plaice, and one of them (phenol UGT) was purified [21]. Phytoestrogens, coumestrol, genistein and biochanin A were highly glucuronidated by carp intestinal microsomes to the same degree as that in rat liver microsomes, indicating that the carp has highly expressed UGT isoform(s) catalyzing the glucuronidation of phytoestrogens in the intestinal musosa.

Most of the metabolites of nonylphenol have been shown to be glucuronic acid conjugates in rainbow trout [15,16]. However, in carp organs, it is difficult for alkylphenols such as nonylphenol and octylphenol to be glucuronidated. Recently, we found that in the rat these alkylphenols were excreted only with difficulty into the bile and easy to be accumulated after glucuronidation in the liver of rat [14]. It is possible that nonylphenol and octylphenol were accumulated in the carp organs as their unconjugated forms. This accumulation of the free form might result in serious physiological consequences, such as aberrations in the reproductive function of carp living in rivers that are polluted with these alkylphenols.

Rat and sheep intestinal UDP-glucuronosyltransferase isoforms were purified and cloned with the designation UGT1A6, UGT1A7, SheUGT1A6 and SheUGT1A7 [22]. The isozymes are able to glucuronidate many xenobiotics and are highly induced by treatment with β-naphthoflavone [23]. Carp intestinal UGT isoform(s) must play important roles in detoxication of phytoestrogens and xenoestrogens, with the exception of alkylphenols. We found that 1-naphthol was highly glucuronidated and that most of the resultant glucuronide was excreted into the mucosal side of the rat intestine [20]. In the present study, we found that bisphenol A was mainly glucuronidated in the carp intestine and excreted into the mucosal side. A recent study showed that most of the bisphenol A injected in the rat was excreted as glucuronide in the feces [24]. If many xenoestrogens and phytoestrogens are excreted into the mucosal side as glucuronides, it presents a very useful system for detoxication of these chemicals at the first barrier.

Acknowledgements

This work was supported by Integrated Research Program for Effects of Endocrine Disrupters on Agriculture, Forestry and Fisheries and Their Action Mechanisms on Domestic Animals and Fishes.

References

[1] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 1993;101:378–84.

[2] Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 1993;132:2279–86.

[3] Soto AM, Justicia H, Wray JW, Sonnenschein C. *p*-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 1991;92:167–73.

[4] White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 1994;135:175–82.

[5] Naylor CG, Mieure JP, Adams WJ, Weeks JA, Castaldi FJ, Ogle LD, Romano RR. Alkylphenol ethoxylates in the environment. J Am Oil Chem Soc 1992;69:695–703.

[6] Blackburn MA, Waldock MJ. Concentrations of alkylphenols in rivers and estuaries in England and Wales. Water Research 1995;29:1623–9.

[7] Mommsen TP, Walsh PJ. Viterogenesis and oocyte assembly. In: Hoar WS, Randall DJ, editors. Fish Physiology, vol. XIA. New York: Academic Press, 1988. p. 347–406.

[8] Colborn T, Clement C. Chemically-induced alterations in sexual and functional development. The wildlife/human connection, vol. XXI. Princeton, N J: Princeton Scientific, 1992.

[9] MacLatchy DL, Van Der Kraak GJ. The phytoestrogen beta-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol 1995;134:305–12.

[10] Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996;104(Suppl 4):741–803.

[11] Purdom CE, Hardiman PA, Bye VJ, Endo NC, Tyler CR, Sumpter JP. Estrogenic effects from sewage treatment works. Chem Ecol 1994;8:275–85.

[12] Yokota H, Iwano H, Endo M, Kobayashi T, Inoue H, Ikushiro S, Yuasa A. Glucuronidation of the environmental oestrogen bisphenol A by an isoform of UDP-glucuronosyltransferase, UGT2B1, in the rat liver. Biochem J 1999;340: 405–9.

[13] Inoue H, Yokota H, Makino T, Onaga T, Yuasa A, Kato S. A Major Metabolite, Bisphenol A-glucuronide in the Rat Bile after Liver Perfusion. Drug Metab Dispos 2001;29:1084–7.

[14] Yokota H, Daidoji T, Inoue H, Kato S, Yuasa A. Glucuronidation and excretion of alkylphenol in rat liver perfusion. 10 th International Workshop on glucuronidation and UDP-glucuronosyltransferases 2001:18.

[15] Thibaut R, Debrauwer L, Rao D, Cravedi JP. Characterization of biliary metabolites of 4-n-nonylphenol in rainbow trout (Oncorhynchus mykiss). Xenobiotica 1998;28:745–57.

[16] Coldham NG, Sivapathasundaram S, Dave M, Ashfield LA, Pottinger TG, Goodall C, Sauer MJ. Biotransformation, tissue distribution, and persistence of 4-nonylphenol residues in juvenile rainbow trout (Oncorhynchus mykiss). Drug Metab Dispos 1998;26:347–54.

[17] Arukwe A, Celius T, Wlther BT, Goksoyr A. Effects of xenoestrogen treatment on *zona radiata* protein and vitellogenin expression in Atlantic salmon (Salmo salar). Aquatic Toxicology 2000;49:159–70.

[18] Imai Y. Reconstituted O-dealkylase systems containing various forms of liver microsomal cytochrome P-450. J Biochem (Tokyo) 1979;86:1697–707.

[19] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.

[20] Inoue H, Yokota H, Kuwahara A, Ogawa H, Kato S, Yuasa A. 1-Naphthol β-D-glucuronide formed intraluminally in rat small intestine mucosa and absorbed into the colon. Life Sciences 1999;36:628–31.

[21] Clarke DJ, George SG, Burchell B. Multiplicity of UDP-glucuronosyltransferases in fish: purification and characterization of a phenol UDP-glucuronosyltransferase from the liver of a marine teleost (Pleuronectes platessa). Biochem J 1992;284:417–23.

[22] Kobayashi T, Tatano A, Yokota H, Onaga T, Watanabe Y, Yuasa A. Small intestinal UDP-glucuronosyltransfera-

se(UGT) UGT1A07: partial purification and cDNA cloning from sheep small intestine. Arch Biochem Biophys 1999;364:143–52.

[23] Kobayashi T, Yokota H, Ohgiya S, Iwano H, Yuasa A. UDP-glucuronosyltransferase UGT1A7 induced in rat small intestinal mucosa by oral administration of β-naphthoflavone. Eur J Biochem 1998;258:948–55.

[24] Pottenger LH, Domoradzki JY, Markham DA, Hansen SC, Cagen SZ, Waechter JM. The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. Toxicol Sci 2000;54:3–18.