Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 344 (2006) 129–133
BBRC
www.elsevier.com/locate/ybbrc

Specific human CYP 450 isoform metabolism of a pentachlorobiphenyl (PCB-IUPAC# 101)

Joseph E. McGraw Sr., Donald P. Waller *

Department of Biopharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, USA

Received 11 March 2006
Available online 29 March 2006

Abstract

Polychlorinated biphenyl IUPAC# 101-PCB 101 (chlorination pattern-2,2′,4′,5,5′) is a common, persistent non-coplanar PCB congener found in the ambient environment but information related to its metabolism in humans is lacking. Previous studies indicate PCB 101 is rapidly metabolized in mammals through CYP 2B and 3A family enzymes. Recently, PCB metabolism through a 2A family isoform in hamsters was also reported. To specifically identify the human CYP 450 isoforms responsible for PCB 101 metabolism, we compared human microsome metabolism to metabolism using several specific recombinant human CYP isoforms. These data characterized selective and extensive metabolism by human CYP 2A6. The product formed was the 4-hydroxy-PCB 101 metabolite (4-hydroxy-2,2′,4′,5,5′) and was the only major metabolite observed in the recombinant and human microsome investigation. This is important information for predicting human specific toxicokinetics of PCBs.

Keywords: PCBs; PCB 101; 2,2′,4′,5,5′- Pentachlorobiphenyl; 4-Hydroxy-PCB 101; 4-Hydroxy-2,2′,4′,5,5′-pentachlorobiphenyl; CYP 2A6; Metabolism; Polychlorinated biphenyls; Recombinant human enzymes; CYP 450

The environmental impact of polychlorinated biphenyls (PCBs) has been studied for over 30 years. They are recognized as one of the most important environmental contaminants by environmental agencies throughout the world including the EPA, ATSDR, WHO, and IJC [1–6]. Numerous studies describing their metabolism in plants and animals as well as disposition in biological and environmental matrices have been published and reviewed [7]. PCB 101 was a major pentachlorinated non-coplanar PCB congener found in the original commercial PCB mixtures and is resistant to environmental degradation. In contrast to other heavily chlorinated PCB congeners, PCB 101 is rapidly metabolized in mammals [7–20]. However, elevated levels of PCB 101 were observed in our cohort of African American women. This could have occurred due to elevated/recent exposure or reduced metabolism. Numerous studies have been conducted in different animal species to investigate metabolite identity, kinetics, and comparative metabolism of PCBs [8,14,19,21–23]. However, data pertaining to human metabolism of PCBs, and PCB 101 specifically, are lacking [1].

PCBs are metabolized by cytochrome P450 (CYP 450) enzymes forming arene oxide and hydroxylated metabolites. Hydroxyl and methylsulfone PCB metabolites have also been recovered from human tissues [24,25]. Persistence of PCB metabolites in tissues varies by parent PCB congener and specific metabolite. Methylsulfone PCBs are exceptionally persistent metabolites formed through epoxide formation via CYP 450 followed by further metabolism through the mercapturic acid pathway to a methylthio metabolite which is oxidized to methylsulfinyl and methylsulfonyl metabolites [26,27]. One of the most persistent methylsulfone PCB metabolites is believed to be derived from PCB 101 [22]. This metabolite has been found in numerous animal species as well as humans.

Few studies of specific PCB congener metabolism by human isoforms have been published. Ariyoshi et al. [17]

* Corresponding author. Fax: +1 312 413 2777.
E-mail address: dpwaller@uic.edu (D.P. Waller).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.03.122

and Schnellman et al. [28] studied PCB 153 metabolism by recombinant microsomes containing human CYP 2B6. The authors found conflicting results for the metabolism of this congener which is known to be resistant to metabolism. Ariyoshi et al. reported metabolites of PCB 153 by human CYP 2B6 with a low reaction rate (6.4 pmol/min/nmol CYP 450) while Schnellman et al. did not report a metabolite. Previous animal studies had shown involvement of CYP 2B and 3A family enzymes in PCB metabolism. However, Koga et al. [29], recently described a CYP 2A family enzyme involved in PCB metabolism in hamsters. Our data identify the specific CYP 450 isozyme responsible for the metabolism of the PCB 101.

Materials and methods

Materials. 2,2′,4,5,5′-PCB IUPAC# 101, 4-hydroxy-2,2′,4,5,5′-PCB 101, and 3-hydroxy-2,2′,4,5,5′-PCB 101 neat standards were obtained from Accustandard Inc. (New Haven, CT). Solvents used were of pesticide grade when available. Microsomal preparations were obtained from BD Biosciences GENTEST (Woburn, MA). Pooled human liver microsomes (HLM's) were obtained with characterization of enzyme activity by CYP 450 specific substrate probes. BD Biosciences Gentest Supersomes are recombinant human CYP isoforms with CYP 450 Reductase created from baculovirus transfected insect cells. Specific human CYP isoforms were obtained with cytochrome b5 where available as cytochrome b5 has been shown to increase enzyme activity. The specific Gentest supersomes obtained were CYP 2A6 + b5, 2B6 + b5, 2C8 + b5, 2C9 + b5, 2C18, 2C19 + b5, 3A4 + b5, 3A5 + b5 (1 nmol P450 in 1 ml) and BD Supermix (CYP 1A2, 2C8, 2C9, 2C19, 2D6, 3A4, 1 nmol P450 in 0.5 ml).

Incubations. PCB 101 hydroxylase activity was determined from screening incubations using Gentest Supersomes and human microsomes followed by kinetic evaluations with specific Gentest Supersomes. An NADPH generating solution was created by mixing 20 μl of 20 mg/ml G6P (1.32 mM), 20 mg/ml NADP (0.5 mM), 13.3 mg/ml $MgCl_2 \cdot 6H_20$ (1.32 mM), and 5 μl of 40 U/ml G6PDH in 5 mM sodium citrate (tribasic) (0.2 U/ml) in a 1.5 ml microcentrifuge tube. Sufficient buffer (Hepes 0.1 M or potassium phosphate 0.05 M) was added to a PCB 101 substrate solution (30 mM) and the NADPH generating system to make 1 ml of reaction volume (Final 0.3 mM PCB 101) and pre-warmed to 37 °C. PCB standard was created by dissolving neat PCB 101 in DMSO. Final PCB 101 concentration was 300 μM in screening incubations while for kinetic incubations it ranged from 0.3 to 300 μM. DMSO was kept at 1% v/v for each incubation. Ten microliters of Supersomes (0.075 mg, 10 pmol) or HLMs (0.2 mg, 76 pmol) was used for screening runs. For kinetic runs, reactions were initiated with 10 μl (0.075 mg,10 pmol) or 5 μl (0.0375 mg, 5 pmol) of supersomes added to warm reaction mixtures and the reaction mixture was incubated for 1 h at 37 °C in a VWR 1100 water bath. The reactions were stopped by adding 100 μl of conc. sulfuric acid and 0.5 ml of a 2:1 chloroform:methanol solution. The entire mixture was then added to a disposable glass culture tube. The microcentrifuge tube was rinsed with 1.5 ml of hexane which was transferred to the culture tube. The glass culture tube was vortexed and the organic fraction was extracted into another glass tube. The rinsing and extraction were repeated two more times. The organic fraction was then blown down under gentle N_2. Samples were reconstituted in 1 ml hexane for GC analysis. Control incubations were conducted for each set of incubations and included Insect Control, BD Supersomes™ Enzymes Cat. No. 456201 (formerly P201), and identical reaction conditions as above except for the addition of concentrated sulfuric acid at time 0.

GC analysis. Analyses were performed using an HP 5890 Series II gas chromatograph with ECD detection. Grade 6 (ultrapure) helium was the carrier gas (flow rate 2 ml/min) with methane/argon makeup. The column was a J&W Scientific DB5 column (30 m × 2.5 μm). The temperature program was as follows; inlet 260 °C, detector 300 °C, initial temp: 150 °C time 4 min, 8 °C/min to 280 °C for 5 min.

GC–MS analysis. Ten incubation mixtures of 2A6 kinetic assays were combined and blown down to ~1 ml hexane. One ml of tetrabutyl ammonium hydroxide (1 M) was added to mixture and 3 ml of purified deionized water. Three microliters of hexane was added to the mixture and vortexed thoroughly. Hexane was extracted off the aqueous phase thrice. Then 1 ml of concentrated sulfuric acid 18 N was added slowly twice to the aqueous phase. After cooling, the aqueous phase was extracted thrice with 1 ml hexane. The organic sample containing hydroxyl PCB metabolites was submitted for GC–MS analysis. Mass spectral analysis was performed using a JEOL GCMate II, LRGCEI mass range 50–550, source temperature 230, acceleration voltage 2500, and mass resolution 500. The GC was an HP 6890 equipped with a DB-5 fused silica DB5 capillary column (30 m × 0.25 mm id, 0.25 μm film thickness) from J&W Scientific Inc. (Folsom CA, USA). The oven temperature was programmed as follows: 80 °C (1 min)–10 °C/min–300 °C (30 min). Helium was used as the carrier gas.

Kinetic evaluations. Incubations as described above were performed with concentrations from 0.3 to 300 μM of PCB 101 along with two runs at 5 pmol of Gentest CYP 2A6 + b5 Supersomes and three runs with 10 pmol of Supersomes under the incubation conditions described above. Calculations for the apparent K_m (±SE) and V_{max} (±SE) were performed by plotting Hanes plots of substrate concentration/reaction rate versus substrate concentration.

Results and discussion

HLMs were evaluated for their ability to produce a PCB 101 metabolite. One predominant metabolite was observed by GC–ECD analysis. CYP 450 Supersomes were then used to determine the specific CYP isozyme(s) responsible for the production of this metabolite. The results of screening individual isoforms for PCB 101 hydroxylase activity are shown in Fig. 1. Gentest supersomes tested also included supermix (CYP 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4) which did not yield any identifiable products. Individual isoforms tested included (CYP 3A4, CYP 3A5, CYP 2A6, CYP 2B6, CYP 2C8, CYP 2C9, CYP 2C18, and CYP 2C19). Three supersomes were identified as producing PCB 101 metabolites. CYP 3A4 and CYP 2C18 produced minimal amounts of a metabolite while the greatest amount of metabolite was produced by the CYP 2A6 isozyme.

The PCB 101 metabolite produced by the CYP 2A6 isozyme was identified as the 4′-hydroxy-2,2′,4,5,5′-pentachlorobiphenyl PCB through retention time matching to standards and confirmation by GC–MS fragmentation patterns. Retention time matching with GC–ECD indicated the PCB 101 metabolite produced by the CYP 3A4 and the CYP 2C18 isozymes were identical to that produced by CYP 2A6.

There is strong agreement between the PCB 101 hydroxylase activities of HLMs versus recombinant CYP 2A6 (see Fig. 2). Based on manufacturer provided reaction rates for the CYP 2A6 specific reaction (coumarin 7-hydroxylase), and the concentrations of P450 in the microsomes, the fractional activity of CYP 2A6 as pmol CYP 2A6 in the HLMs is 5%. Assuming CYP 2A6 represents 5% of the picomoles in our HLM reaction rate (in pmol/min/pmol CYP 2A6), reaction rates for CYP 2A6 in HLMs and CYP 2A6

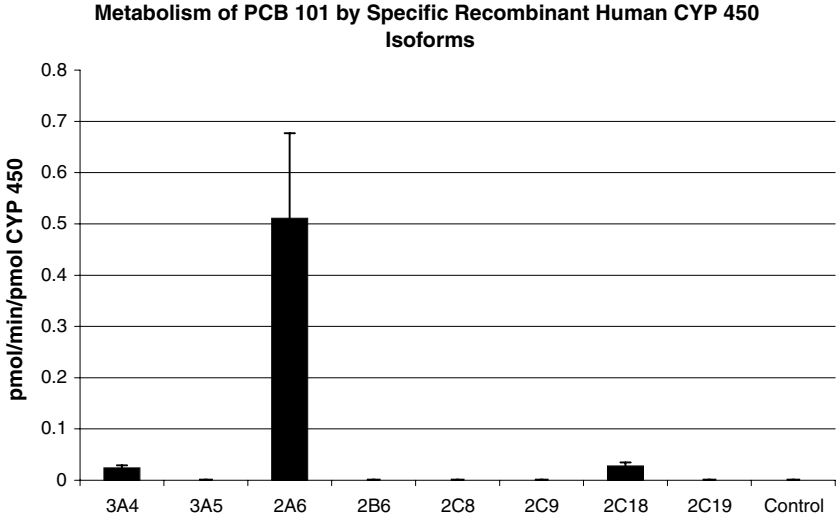

Fig. 1. Specific human CYP 450 isoforms (10 pmol recombinant Gentest microsomes) were incubated with PCB 101 (300 μM) with a NADPH regeneration system. Metabolites were analyzed by GC with ECD detection.

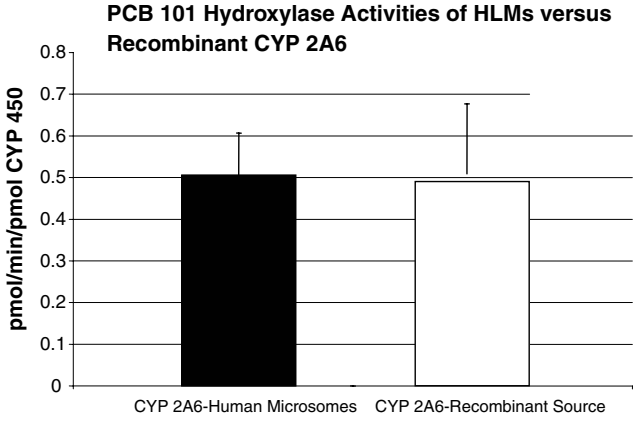

Fig. 2. Human CYP 2A6 isoform (10 pmol recombinant Gentest microsomes) and pooled Human Liver microsomes (HLMs) (76 pmol) were incubated with PCB 101 (300 μM) with a NADPH regeneration system. Metabolites were analyzed by GC with ECD detection. Activity of the HLMs is corrected according to reaction phenotyping for 2A6 (5%) in the total cytochrome P450 activity.

specific Gentest Supersomes were 0.51(±0.10) vs. 0.51(±0.17) pmol/min/pmol CYP 2A6, respectively. These values do not differ by statistical testing (t test, $p < 0.01$). CYPs 3A4 and CYP 2C18 also metabolize PCB 101 although to much less an extent than CYP 2A6 (Fig. 1). Even though CYP 3A4 is a major hepatic enzyme in liver and intestine [30], the identical rate of PCB 101 metabolism for recombinant 2A6 and in HLMs attributable to 2A6 indicates PCB 101 is a poor substrate for CYPs other than human CYP 2A6. Coumarin 7-hydroxylase is considered quite specific to CYP 2A6. The data shown indicate PCB 101 metabolism is of the same order of specificity.

In vivo PCB 101 metabolism will most likely occur primarily via the isoform CYP 2A6. The contribution of other isozymes to PCB 101 metabolism is negligible, even at the high substrate concentrations in our evaluations. Therefore, other isoforms would contribute very little to the overall metabolism of PCB 101 at the very low PCB concentrations found in human tissues.

A Michaelis–Menten kinetic plot of human CYP 2A6 Gentest microsome PCB 101 conversion to 4-OH PCB 101 is presented in Fig. 3. Two concentrations (5 and 10 pmol) of microsomal protein were evaluated and demonstrated linearity of metabolite produced over the range tested. The data appear to follow Michaelis–Menten kinetics.

The K_m and the V_{max} of PCB 101 metabolism by CYP 2A6 were determined using human CYP 2A6 supersomes. The Hanes plot presented in Fig. 4 is a linearized representation of the data fit to the Michaelis–Menten model. The strong R^2 value (>0.99) indicates good agreement of data with a Michaelis–Menten model. The apparent K_m [34(±5) μM] is the X-intercept. The slope is the inverse V_{max} apparent [0.65(±0.04) pmol/min/pmol CYP 450].

The results of this paper are consistent with those of Koga et al. who showed remarkably similar results for

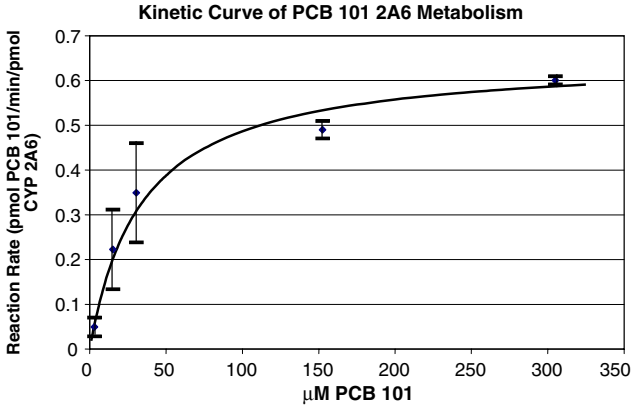

Fig. 3. The curve above represents PCB 101 conversion to 4-OH PCB 101. Mean reaction rates are bracketed by ±one standard deviation.

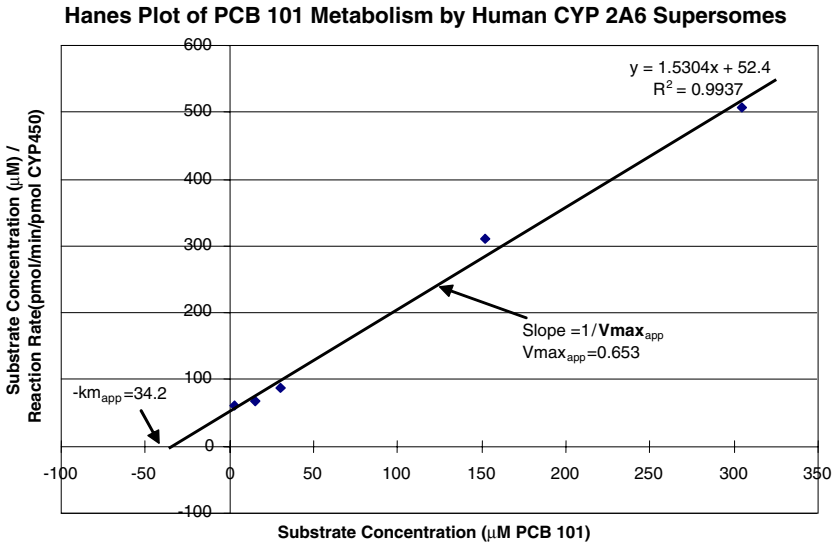

Fig. 4. Data are based on incubations with 2A6 supersomes. The Hanes plot is a linearized representation of the data fit to the Michaelis–Menten model. The R^2 value (>0.99) indicates good agreement of data with a Michaelis–Menten model.

PCB 52 (2,5,2′,5′-tetrachlorobiphenyl) metabolism by hamster liver CYP 2A8 [29]. PCB 52 is homologuous to PCB 101 with respect to metabolism as it shares the same chlorination pattern of the ring which is selectively metabolized in PCB 101. The 2,5 chlorination ring pattern is metabolized because its vicinal meta, para carbons are open to oxidation by CYP 450 through an epoxide intermediate. CYP 2A8 is a known homologue of human CYP 2A6 with similar metabolism of nicotine to human 2A6. Similar to our data, Koga et al. showed preferential formation of the 4-hydroxy metabolite, which they believed was formed through a 3-4 arene oxide intermediate. They showed a decrease in product formation by addition of serum, which inhibits arene oxide intermediate formation. Arene oxide derived metabolites have also been isolated from the urine of primates dosed with PCB 52 [31]. Future studies may provide further insight into the mechanism of human CYP 2A6 metabolism of PCB 101.

Based on animal experiments reported and reviewed in the literature, CYPs from the 3A and 2B family were initially suspected as being responsible for PCB 101 metabolism [7]. A previous study also showed human CYP 2B6 metabolized PCB congener 153 [17]. However, screening and extensive evaluations of enzyme incubations clearly indicated CYP 2B6 was not responsible for the metabolism of PCB 101. Screening incubation data and individual kinetic experiments presented in this manuscript strongly demonstrate the metabolism of PCB 101 by CYP 2A6 is specific and extensive.

Reduced nicotine clearance in African Americans has recently been associated with altered CYP 2A6 metabolism. We have also observed elevated PCB 101 levels in the Chicago Great Lakes African American Cohort and other published cohorts with African ancestry. These observations are consistent with reduced metabolism of CYP 2A6 substrates in African Americans.

These data identify recombinant human CYP 2A6 as the human CYP isoform responsible for the metabolism of PCB 101. This CYP was not expected to metabolize PCB 101 based upon previous animal studies which demonstrated PCB 101 metabolism by 2B and 3A family enzymes. The lack of any appreciable metabolism by other CYP isoforms provides strong evidence that the human metabolism of PCB 101 is exclusively mediated by CYP 2A6. This is the first study to match a specific human CYP isoform responsible for the selective metabolism of an individual PCB congener.

Acknowledgments

This investigation was conducted in a facility constructed with support from Grant C06RR15482 from the NCRR NIH. A portion of the work was funded by CDC/ATSDR Great Lakes Program Grant H75/ATH598336-10.

References

[1] ATSDR, Toxicological Profile For Polychlorinated Biphenyls (PCBs), US Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, GA, 2000.
[2] ATSDR, 2001 CERCLA Priority List Hazardous Substances, Agency for Toxic Substances and Disease Registry, 2001.
[3] USEPA, Second National Report on Human Exposure to Environmental Chemicals, Environmental Protection Agency, Baltimore, MD, USA, 2003.
[4] IJC, 11th Biennial Report—Great Lakes Water Quality, IJC 11th Biennial Report—Great Lakes Water Quality, 2002.
[5] WHO, WHO/WHO Sites/Food Safety/Chemical Risk in Food, WHO/WHO Sites/Food Safety/Chemical Risk in Food, 2005.
[6] H.E. Hicks, The Great Lakes: a historical overview, Toxicol. Ind. Health 12 (1996) 303–313.
[7] L. Hansen, The Ortho side of PCBs: Occurrence and Disposition, in, Kluwer Publishing, Norwell MA 02061, 1999, pp. Appendix Table 2. Pgs. 205–210.

[8] H.L.E. Klasson-Wehler, B.O. Lund, 2,2′,4,5,5′-Pentachlorobiphenyl: comparative metabolism in mink (Mustela vision) and mouse, Chem. Res. Toxicol. 9 (1996) 1340–1349.
[9] B.D. Preston, J.A. Miller, E.C. Miller, Non-arene oxide aromatic ring hydroxylation of 2,2′,5,5′-tetrachlorobiphenyl as the major metabolic pathway catalyzed by phenobarbital-induced rat liver microsomes, J. Biol. Chem. 258 (1983) 8304–8311.
[10] B.D. Preston, J.A. Miller, E.C. Miller, Reactions of 2,2′,5,5′-tetrachlorobiphenyl 3,4-oxide with methionine, cysteine and glutathione in relation to the formation of methylthio-metabolites of 2,2′,5,5′-tetrachlorobiphenyl in the rat and mouse, Chem. Biol. Interact. 50 (1984) 289–312.
[11] Y. Masuda, H. Kuroki, K. Haraguchi, A. Schecter, O. Papke, The condition of PCBs and PCDFs in the blood of Yusho patients 20 years after the onset, Fukuoka Igaku Zasshi 88 (1997) 149–156.
[12] K. Haraguchi, Y. Masuda, Tissue distribution of methylsulfonyl metabolites derived from Kanechlor 400 in mice, Fukuoka Igaku Zasshi 88 (1997) 157–161.
[13] K. Haraguchi, Y. Kato, Y. Masuda, R. Kimura, Metabolism of 3,3′,4,4′-tetrachlorobiphenyl via sulphur-containing pathway in rat: liver-specific retention of methylsulphonyl metabolite, Xenobiotica 27 (1997) 831–842.
[14] K. Haraguchi, Y. Kato, R. Kimura, Y. Masuda, Comparative study on formation of hydroxy and sulfur-containing metabolites from different chlorinated biphenyls with 2,5-substitution in rats, Drug Metab. Dispos. 25 (1997) 845–852.
[15] K. Haraguchi, K. Ito, H. Kotaki, Y. Sawada, T. Iga, Prediction of drug-induced catalepsy based on dopamine D1, D2, and muscarinic acetylcholine receptor occupancies, Drug Metab. Dispos. 25 (1997) 675–684.
[16] N. Ariyoshi, N. Koga, K. Oguri, H. Yoshimura, Metabolism of 2,4,5,2′,4′,5′-hexachlorobiphenyl with liver microsomes of phenobarbital-treated dog; the possible formation of PCB 2,3-arene oxide intermediate, Xenobiotica 22 (1992) 1275–1290.
[17] N. Ariyoshi, K. Oguri, N. Koga, H. Yoshimura, Y. Funae, Metabolism of 2,5,2′ -trichloro-, 2,5,2′,5′ -tetrachloro-, and 2,4,5,2′,5′ -pentachlorobiphenyl in rat hepatic microsomal systems, Biochem. Biophys. Res. Commun. 212 (1995) 455–460.
[18] S.M. Ghiasuddin, R.E. Menzer, J.O. Nelson, Metabolism of 2,5,2′ -trichloro-, 2,5,2′,5′ -tetrachloro-, and 2,4,5,2′,5′ -pentachlorobiphenyl in rat hepatic microsomal systems, Toxicol. Appl. Pharmacol. 36 (1976) 187–194.
[19] Y. Kato, K. Haraguchi, T. Yamazaki, R. Kimura, N. Koga, S. Yamada, M. Degawa, The decrease in level of serum thyroxine by 2,2′,4,5,5′-pentachlorobiphenyl in rats and mice: no correlation with formation of methylsulfonyl metabolites, Drug Metab. Dispos. 33 (2005) 1661–1665.
[20] M. Berlin, J. Gage, S. Holm, Distribution and metabolism of 2,4,5,2′, 5′-pentachlorobiphenyl, Arch. Environ. Health 30 (1975) 141–147.
[21] K. Haraguchi, Y. Kato, N. Koga, M. Degawa, Species differences in the tissue distribution of catechol and methylsulphonyl metabolites of 2,4,5,2′,5′-penta- and 2,3,4,2′,3′,6′-hexachlorobiphenyls in rats, mice, hamsters and guinea pigs, Xenobiotica 35 (2005) 85–96.
[22] K. Haraguchi, Y. Kato, R. Kimura, Y. Masuda, Tissue distribution of methylsulfonyl metabolites derived from 2,2′,4,5,5′-penta- and 2,2′,3,4′,5′,6-hexachlorobiphenyls in rats, Arch. Environ. Contam. Toxicol. 37 (1999) 135–142.
[23] K. Haraguchi, Y. Kato, N. Koga, M. Degawa, Metabolism of polychlorinated biphenyls by Gunn rats: identification and serum retention of catechol metabolites, Chem. Res. Toxicol. 17 (2004) 1684–1691.
[24] B. Fangstrom, M. Athanasiadou, P. Grandjean, P. Weihe, A. Bergman, Hydroxylated PCB metabolites and PCBs in serum from pregnant Faroese women, Environ. Health Perspect. 110 (2002) 895–899.
[25] C.D. Sandau, P. Ayotte, E. Dewailly, J. Duffe, R.J. Norstrom, Pentachlorophenol and hydroxylated polychlorinated biphenyl metabolites in umbilical cord plasma of neonates from coastal populations in Quebec, Environ. Health Perspect. 110 (2002) 411–417.
[26] J.E. Bakke, A.L. Bergman, I. Brandt, P. Darnerud, C. Struble, Metabolism of the mercapturic acid of 2,4′,5-trichlorobiphenyl in rats and mice, Xenobiotica 13 (1983) 597–605.
[27] J.E. Bakke, A.L. Bergman, G.L. Larsen, Metabolism of 2,4′, 5-trichlorobiphenyl by the mercapturic acid pathway, Science 217 (1982) 645–647.
[28] R.G. Schnellmann, C.W. Putnam, I.G. Sipes, Metabolism of 2,2′,3,3′,6,6′-hexachlorobiphenyl and 2,2′,4,4′,5,5′-hexachlorobiphenyl by human hepatic microsomes, Biochem. Pharmacol. 32 (1983) 3233–3239.
[29] N. Koga, N. Kikuichi, T. Kanamaru, N. Ariyoshi, K. Oguri, H. Yoshimura, Hamster liver cytochrome P450 (CYP2A8) as a 4-hydroxylase for 2,5,2′,5′-tetrachlorobiphenyl, Biochem. Biophys. Res. Commun. 225 (1996) 685–688.
[30] G.K. Dresser, J.D. Spence, D.G. Bailey, Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition, Clin. Pharmacokinet. 38 (2000) 41–57.
[31] I.C. Hsu, J.P. van Miller, J.L. Seymour, J.R. Allen, Urinary metabolites of 2, 5, 2′, 5′-tetrachlorobiphenyl in the nonhuman primate, Proc. Soc. Exp. Biol. Med. 150 (1975) 185–188.