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ORIGINAL RESEARCH

Sensitive determination of bisphenol A and alkylphenols by high performance liquid chromatography with pre-column derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole

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ABSTRACT: A new and sensitive high-performance chromatographic method for the determination of bisphenol A and 8 alkylphenols with fluorescence detection is reported. Each phenol was derivatized by reaction with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole at 40°C for 60 min. The fluorescence derivatives were separated on a Wakosil 5C18 column (4.0 i.d. × 300 mm, 5 μm) with methanol:water (10:90) as mobile phase (detection wavelength: λ_{ex} 336 nm, λ_{em} 440 nm). The detection limits were in the range of 0.1–10.0 pg/mL in serum. The calibration graphs were linear to 1.0 μg/mL. The relative standard deviations were 7.2–8.9%, respectively. The proposed method was applied to the determination of bisphenol A in mother and infant rat serum. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

The bisphenol A and alkyl phenols have been widely reported as endocrine disruptors (EDs) (Rivas *et al.*, 1997; Ashby and Tinwell, 1998; Odum *et al.*, 1999). Gas chromatography (GC) (Kvistad *et al.*, 1998), gas chromatography–mass spectrometry (GC-MS) (Jahr, 1998; del Olmo *et al.*, 1997; Markham *et al.*, 1998), high performance liquid chromatography (HPLC) (Markham *et al.*, 1998; Rudel *et al.*, 1998) have been used for determination of bisphenol A and alkylphenols. We have reported a pre-column HPLC method for alkyl alcohols (Katayama *et al.*, 1991), 21-hydroxycorticosteroids (Katayama *et al.*, 1993), estrogen (Katayama *et al.*, 1993; Mukaida *et al.*, 1995; Katayama *et al.*, 1998) involving the use of 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) with fluorescence detection. The proposed HPLC methods had similar sensitivity to GC-MS, enabling the determination of each analyte at the picogram level. They required only a simple one-step extraction for sample preparation and simple apparatus (HPLC basic system with fluorescence detector).

The aim of our research was in the monitoring of bisphenol A and alkyl phenol for diagnosis of transfer of phenols from mother to infant and the relationship between liver function, weight and hormone (estradiol and testosterone) values. Therefore, we needed to develop a sensitive HPLC method for the determination of bisphenol A and alkyl phenols in biological fluid for use in the clinical laboratory.

In this paper, we describe a pre-column HPLC method for the determination of bisphenol A and alkylphenol with CDB (Fig. 1) as the fluorescent derivatization reagent.

EXPERIMENTAL

Samples and reagents. Bisphenol A and alkylphenols (4-*sec*-butylphenol, 2-*tert*-butylphenol, 3-*tert*-butylphenol, 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*tert*-pentylphenol, 4-*n*-hexylphenol, and 4-*n*-heptylphenol) were reagent grade for ED analysis (Wako Pure Chemical Co., Japan). 2-(4-Carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) was synthesized as previously reported

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Abbreviations used: CDB, 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole; ED, endocrine discriptor; IDC, 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate.

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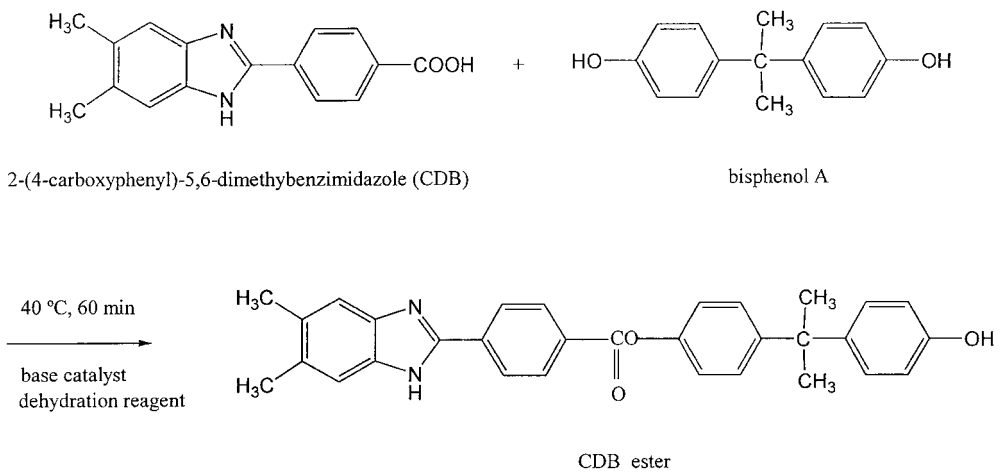

Figure 1. Reaction scheme of CDB and bisphenol A.

(Katayama *et al.*, 1991). Each reagent was prepared as follows: CDB (1.0% w/v) stock solution—10 mg of CDB were dissolved in 1.0 mL of pyridine; CDB (0.02% w/v) solution,—20 μL of CDB stock solution were added to 4-piperidinopyridine (Aldrich, Milwaukee, WI, USA) and then diluted to 1.0 mL with acetonitrile; IDC (2.0% w/v) solution—20 mg of 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate (IDC, Wako Pure Chemical Co., Japan) were dissolved in 1.0 mL of acetonitrile. 4 Hydroxybenzoic acid *sec*-butyl ester (Tokyo Kasei Co, Japan) was used as internal standard. All solvents used were HPLC grade.

Blood samples. Sprague–Dawley strain rats (NRC Haruna, Gunma, Japan) were used. The animals were maintained under controlled conditions (22 ± 2°C, 55 ± 5% humidity, 12 h light/dark cycle, light from 06:00 to 18:00) and were given laboratory chow (CE-2, Nippon CREA) *ad libitum*. Female rats were housed with males, and the morning when a vaginal plug was found was designated day 1 of gestation. Pregnant females were housed individually and treated with 0.2, 2, 20 and 200 μg mL^{-1} bisphenol A (Aldrich, Milwaukee, WI, USA) dissolved in 0.05% w/v ethanol solution (vehicle) using glass bottles from the day 1 of gestation until the day of sacrifice. Control animals were given 0.05% w/v ethanol solution. The pregnant females were given bisphenol A solution or vehicle *ad libitum*. Pregnant rats were killed by cervical dislocation at the day 22 of gestation respectively and the fetuses delivered by Caesarean operation. Blood was collected by heart puncture in mothers and by decapitation in fetuses. Blood samples were centrifuged immediately at 1500 ***g*** for 15 min at 4°C. The serum was subsequently isolated and stored at −20°C in Serum Tube (Sumitomo Bakelite, Tokyo, Japan) until use.

Conditions for pre-column HPLC. Conditions were: HPLC pump, Shimadzu LC-6A liquid chromatograph (Shimadzu, Japan); column, Wakosil $5C_{18}$ (4.0 mm i.d. × 300 mm, 5 μm, Wako Pure Chemicals, Japan); injection volume, 20 μL; column temperature, ambient (ca. 23°C); detector, Shimadzu RF-535A fluorescence spectromonitor (excitation wavelength = 336 nm, emission wavelength = 440 nm); mobile phase, methanol:water (90:10); flow-rate, 0.7 mL/min; integrator, Shimadzu CR-5A chromatopac.

HPLC conditions for native fluorescence detection. Conditions were: HPLC pump, Shimadzu LC-6A liquid chromatograph (Shimadzu, Japan); column, Capcell Pak ODS SG 120 A (4.0 mm i.d. × 150 mm, 5 μm, Shiseido, Japan); sample solvent, 20 μL; column temperature, ambient (ca. 23 °C); detector, Shimadzu RF-535A fluorescence spectromonitor (excitation wavelength = 275 nm, emission wavelength = 315 nm); mobile phase, methanol:water (70:30); flow-rate, 0.7 mL/min; integrator, Shimadzu CR-5A chromatopac.

Extraction method. A 100 μL sample of rat serum from mother or infant was placed into a 3.4 mL serum tube (Sumitomo Bakelite Co., Japan) and 400 μL of water were added. The mixture was extracted with 500 μL of acetonitrile for 5 min. An aliquot of 400 μL of acetonitrile was taken and dried by anhydrous sodium sulfate. Then, 30 μL of CDB solution and 30 μL of IDC solution were added to 400 μL of the dried acetonitrile layer. The mixture was heated at 40°C for 60 min. A 20 μL sample of the derivatized solution was injected into HPLC apparatus.

RESULTS

Derivatization conditions

To optimize the derivatization conditions, the choice of condensing reagent, base catalyst and solvent for derivatization was studied as previously reported manner (Katayama *et al.*, 1998). Per the results, 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate (IDC) as condensing reagent, 4-piperidinopyridine as base catalyst and acetonitorile as derivatizing solvent were selected. Each reagent concentration was set as follows: CDB solution, 0.02% w/v; IDC solution, 2.0% w/v; 4-piperidinopyridine, 0.01% w/v (10 mg/mL). The highest detector response was obtained at 40°C over 60 min. The derivatization temperature was set at 40°C for 60 min. The derivatization products from phenols were esters (Katayama *et al.*, 1993). It was suspected

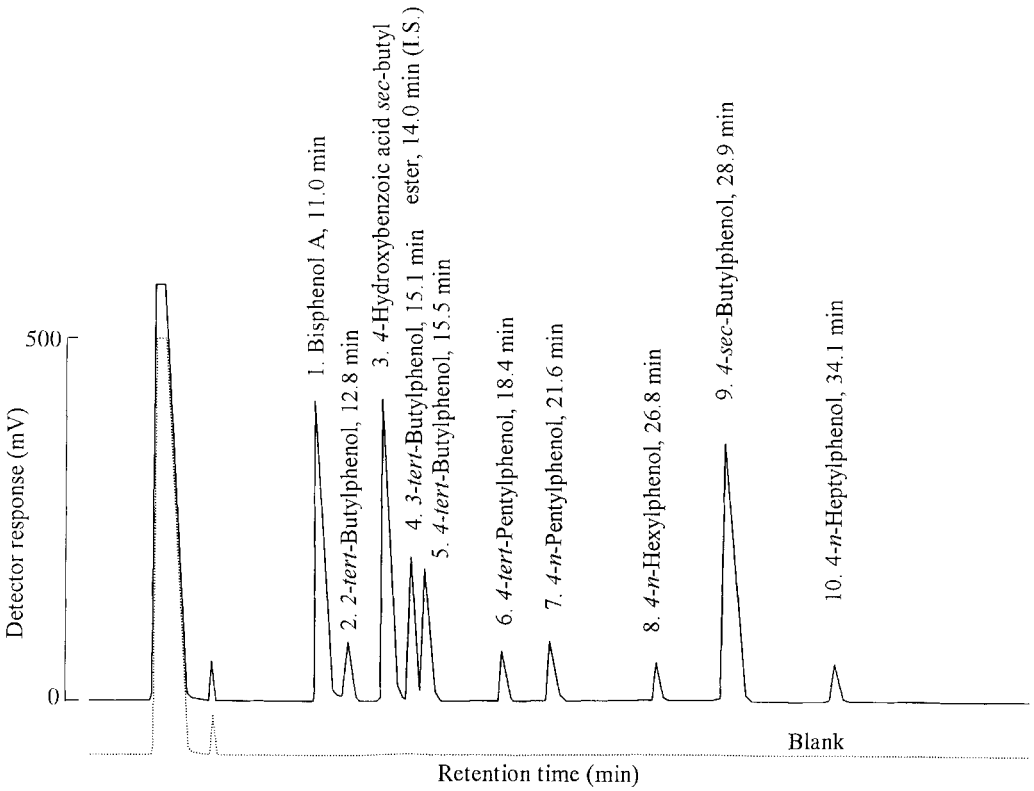

Figure 2. Chromatogram of bisphenol A and alkylphenols. Each pehnol was derivatized by 2-(4-hydroxyphenyl)-5,6-dimethylphenol (50 ng/ml).

that bisphenol A gave mono or disubstitued fluorescent products. We took fluorescent product separated by proposed pre-column LC and the -OH group was tested by ferric chloride solution. As the result, the -OH group was identified. It was thought that CDB formed the CDB-IDC-dimethylaminopyridine conjugate initially, and then phenol reacted with the conjugate. Therefore, monosubstituted derivatization product from bisophenol A was formed by sterific hindrance (Ziegler and Berger, 1979).

Chromatograms

The chromatograms of spiked (50 ng/mL) bisphenol A and alkylphenols (4-*sec*-butylphenol, 2-*tert*-butylphenol, 2-*tert*-butylphenol, 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*tert*-pentylphenol, *p*-*n*-hexylphenol, 4-*n*-heptylphenol) in serum are shown in Fig. 2. Nine samples were determined within 35 min. The determination limits (bisphenol Al, 0.1 pg/mL; alkylphenols, 0.7–10.0 pg/mL) are shown in Table 1. The proposed HPLC method

Table 1. Precision and linearity of bisphenol A and alkyl phenols ($n = 6$) in serum

Phenols	Precision (C.V.%), 50 ng/ml		L.D.[a] (pg/ml)	Linearity[b,c]	Recovery (%) (50 ng/ml)
	Intra-day	Inter-day			
Bisphenol A	7.4	7.8	0.1	$r = 0.9999$, $y = 0.742\,x - 0.011$	91.4 ± 8.1
4-*sec*-Butylphenol	7.9	8.0	0.7	$r = 0.9998$, $y = 0.682\,x - 0.009$	90.6 ± 8.0
2-*tert*-Butylphenol	7.8	8.0	2.0	$r = 1.0000$, $y = 0.020\,x - 0.001$	91.2 ± 7.3
3-*tert*-Butylphenol	7.2	7.4	1.0	$r = 0.9999$, $y = 0.068\,x - 0.003$	90.3 ± 7.6
4-*tert*-Butylphenol	7.4	7.5	1.0	$r = 0.9999$, $y = 0.070\,x - 0.001$	90.1 ± 7.3
4-*n*-Pentylphenol	7.2	7.3	3.0	$r = 1.0000$, $y = 0.034\,x + 0.003$	89.8 ± 7.0
4-*tert*-Pentylphenol	8.5	8.9	3.0	$r = 0.9999$, $y = 0.020\,x - 0.001$	90.6 ± 7.6
4-*n*-Hexylphenol	8.2	8.3	5.0	$r = 1.0000$, $y = 0.018\,x - 0.001$	90.1 ± 7.8
4-*n*-Heptylphenol	7.2	7.6	10.0	$r = 0.9999$, $y = 0.581\,x + 0.059$	90.9 ± 8.1

[a] Limit of detection (S/N = 5).
[b] y = peak area: x = concentration (ng/ml).
[c] Linearities were calculated from L.D. to 1.0 μg/ml samples.

Table 2. Bisphenol A concentration in mother and infant rat

Dose	Mother	Concentration (ng/ml)	Infant	Concentration (ng/ml)
Control	RM1	N.D.[a]	R1	N.D.
			R2	N.D.
			R3	N.D.
			R4	N.D.
	RM2	N.D.	R5	N.D.
			R6	N.D.
			R7	N.D.
			R8	N.D.
0.2 μg/ml	RM3	27.1 ± 1.5	R9	10.3 ± 0.9
			R10	12.5 ± 1.1
			R11	9.5 ± 1.9
	RM4	74.7 ± 4.5	R12	11.7 ± 2.9
			R13	38.6 ± 9.8
			R14	19.4 ± 2.6
2.0 μg/ml	RM5	55.3 ± 1.5	R15	12.1 ± 0.5
			R16	2.6 ± 0.2
			R17	N.D.
	RM6	72.9 ± 4.4	R18	88.1 ± 14.1
			R19	44.7 ± 10.3
			R20	101.9 ± 10.9
20 μg/ml	RM7	99.1 ± 7.7	R21	100.7 ± 9.7
			R22	113.6 ± 15.9
			R23	22.4 ± 2.6
	RM8	38.5 ± 8.3	R24	34.2 ± 3.2
			R25	40.2 ± 4.0
			R26	74.0 ± 11.3
200 μg/ml	RM9	33.8 ± 1.5	R27	82.2 ± 12.1
			R28	27.1 ± 3.0
			R29	74.0 ± 9.5
	RM10	86.9 ± 1.6	R30	41.3 ± 6.3
			R31	13.3 ± 2.3
			R32	89.1 ± 1.6

[a] No response on detector.

was more sensitive than previous reports by photometric detection (Rudel *et al.*, 1998) and native fluorescence detection (Markham *et al.*, 1998). The calibration graphs were linear to 1.0 μg/mL. The relative standard deviations were 7.2–8.5% (intra-day) and 7.3–8.9% (inter-day). The recoveries of bisphenol A and alkylphenols from serum were in the range of 89.8–91.4%.

Comparison with other HPLC

The proposed pre-column HPLC was compared to other HPLC with native fluorescence detection (see Experimental section). Thirty samples (10–500 ng/mL) were determined by both methods. The correlation coefficient was 1.000 and regression equation was $y = 1.002 + 0.742$ (95.0% coincidence limits for slope, $1.001 \leq \alpha \leq 1.003$, and the intercept, $0.395 \leq \beta \leq 1.090$), where y and x are concentrations, in ng/mL, obtained by the proposed pre-column HPLC and HPLC by native fluorescence detection, respectively.

Monitoring bisphenol A in rat samples

The concentration of bisphenol A in rat plasma was monitored by the proposed HPLC method. Various concentrations of bisphenol A (0.2–200 μg/mL) were administrated in drinking water for 22 days. Blood from mother and infant were analyzed. The results are summarized in Table 2. The bisphenol A concentrations in the 0.2 μg/mL group of mothers were 27.1–74.7 ng/mL, while in the infants they were 9.5–38.6 ng/mL. The bisphenol A concentrations in infants were less than 50% of those in mothers. On the other hand, in the 20 and 200 μg/mL administration groups, bisphenol A concentrations in mothers were 33.8–99.1 ng/mL. There were variant bisphenol A concentrations in all administration groups. The bisphenol A concentrations (20 and 200 μg/mL dose to mother) in infants were 22.4–113.6 μg/mL. The concentrations in mothers and infants were the same in both groups. The 2.0 μg/mL dose group showed the same results.

There have some reports on EDs [(genistein), plant estrogen (Adlercreutz *et al.*, 1999) and octylphenol (Certa *et al.*, 1996)], and concentrations in blood among

mothers and infants were of similar levels. In our study, the 20 and 200 μg/mL dose groups showed the same results, but in the low dose mother group, lower bisphenol A concentrations were found in infants. The reasons for this may be differences in metabolic speed and pathway [metabolite to glucuronide and sulfate (Adlercreutz *et al.*, 1999; Certa *et al.*, 1996)] and protein binding (Arnold *et al.*, 1996). Further studies are needed to understand the transportation of bisphenol A between mother and infant.

DISCUSSION

We have developed a new sensitive pre-column HPLC method for the determination of bisphenol A and alkylphenols with fluorescence detection. The proposed HPLC method could determine 0.1–10.0 pg/mL bisphenol A and alkylphenols. It was more sensitive compared to HPLC by photometric detection (Rudel *et al.*, 1998), native fluorescence detection (Markham *et al.*, 1998) and with the same sensitivity compared to GC-MS methods (Jahr, 1998; del Olmo *et al.*, 1997; Markham *et al.*, 1998). Good recoveries (89.9–91.4%) and good correlation coefficient (1.000) with other HPLC methods were obtained. The proposed pre-column HPLC method was applied to study bisphenol A transportation from mother to infant. The concentrations between mother and infant were the same in the 20–200 μg/mL administration groups. These results were consistent with the report of genistein (Adlercreutz *et al.*, 1999) and octylphenol (Certa *et al.*, 1996). The infants in low administion group (0.2 μg/mL) gave half the level of mothers. It was hypothesized that the differences were due to metabolite pathway, speed of metabolism and protein binding.

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