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High-performance liquid chromatographic analysis of bisphenol A and 4-nonylphenol in serum, liver and testis tissues after oral administration to rats and its application to toxicokinetic study

Quanwei Xiao, Yuanqian Li *, Huaxue Ouyang, Peiyu Xu, Desheng Wu

West China School of Public Health, Sichuan University, Chengdu, Sichuan 610041, China

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Abstract

A sensitive and simple method based on solid-phase extraction (SPE) and HPLC with fluorescence detection for the determination of bisphenol A (BPA) and 4-nonylphenol (4-NP) in rat serum, liver and testis tissues has been developed. The chromatographic conditions consisted of a C18 column and mobile phase composition of acetonitrile and water with flow rate of 1.0 ml/min. The fluorescence detection was performed at excitation and emission wavelengths of 227 nm and 313 nm, respectively. Under these conditions, BPA and 4-NP were well separated and showed good linearities in the ranges of 0.01–50.0 μg/ml for BPA and 0.15–150.0 μg/ml for 4-NP with correlation coefficients greater than 0.999. The detection limits of serum and tissue samples were 2.8 ng/ml and 1.4 ng/g for BPA and 5.6 ng/ml and 2.8 ng/g for 4-NP at a signal-to-noise ratio (S/N) of 3. The intra-assay and the inter-assay precisions were better than 11.4%. Recoveries of BPA and 4-NP were 78.6–95.0% and 80.2–93.4%, respectively. The proposed method was applied to a toxicokinetic study of BPA and 4-NP including individual and combined oral administration to rats. The results showed that 4-NP remarkably altered the toxicokinetic parameters of BPA in testis, while parameters of BPA were not obviously altered in serum and liver under the experimental conditions investigated. On the other hand, there was no significant difference in the toxicokinetics of 4-NP when administered with BPA.

Keywords: HPLC; Environmental estrogens; Bisphenol A; 4-Nonylphenol; Solid-phase extraction; Rat serum and tissue; Toxicokinetics

1. Introduction

Xenoestrogens are environmental chemicals with the potential to affect the normal endocrine functions of humans and wildlife by interfering with effects of endogenous sex hormones and thereby adversely affecting their reproduction and development [1,2]. 4-4′-Isopropylidene diphenol (bisphenol A, BPA), one of endocrine estrogenic compounds, is a primary raw material used for the production of polycarbonates and epoxy resins [3] and has widespread use in the production of food packaging materials and food containers, from which BPA could migrate to food and enter into body. 4-Nonylphenol (4-NP), one of alkylphenols, is a breakdown product of alkylphenol polyethoxylates (APEs), an important kind of non-ionic surfactants that are widely used in many detergent formulations and plastic products for industrial and domestic use. 4-NP can mimic the action of estrogens by binding to estrogen receptors and may contribute to adverse effects in wildlife and humans. 4-NP which can be formed by metabolism of its ethoxylates in vertebrates and microorganisms, is released into the aquatic environment and has an enhanced resistance towards biodegradation and ability to bioaccumulate. In addition, the plastic products used in the process of food handling and packaging usually have detectable amounts of 4-NP which may migrate to high fat food [4–6].

Many xenoestrogens exist simultaneously in the environment, and thus, exposure to mixtures by various routes needs to be taken into account. For instance, BPA and 4-NP can enter the body via the gastrointestinal tract as food contaminants. To date, study on toxicokinetic interaction between BPA and 4-NP has not been reported. In order to study the toxicokinetics of BPA and 4-NP in rat serum and tissue, it is desirable to develop a sensitive, easily performable method for the quantitative determination of BPA and 4-NP in rat serum and tissue samples.

* Corresponding author. Tel.: +86 28 85501301; fax: +86 28 85501275.
E-mail address: Liyuanqian@hotmail.com (Y. Li).

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There are many different analytical methods for the determination of BPA and 4-NP in aquatic environment, plastics and soil, including GC–MS, LC–MS and HPLC–FLD [7–11]. However, only a few analytical methods for the determination of BPA or 4-NP in biological samples were reported. Miyakoda et al. [12] reported that BPA in rat tissue was analyzed with GC–MS after derivatization. 4-NP in rat tissue was determined by LC–MS after derivatization with pentafluorobenzylbromide (PFB-Br) [13] or was extracted with concurrent steam-distillation and solvent extraction and then determined by HPLC with UV detector [14]. However, the sample extraction and derivatization were tedious and time-consuming. To our knowledge, no publication has described the simultaneous determination of BPA and 4-NP in rat tissues.

Pretreatment of the biological samples prior to instrumental analysis is a very important step. In our previous work, a liquid–liquid extraction technique was used in the pretreatment of rat tissue for simultaneous determination BPA and 4-NP by HPLC [15]. However, liquid–liquid extraction of the biological samples requires a large amount of organic solvents and is rather time-consuming. Recently, solid-phase extraction (SPE) technique has been proven to be a useful means for analysis of biological samples, because it is easily performable and needs a small amount of organic solvent. The purpose of this study was to develop a solid-phase extraction technique using C18 cartridge for extraction and clean-up of biological samples and a sensitive HPLC analytical method with direct fluorescence detection for the simultaneous determination of BPA and 4-NP in rat serum and tissues after oral administration as well as apply to their toxicokinetics.

2. Experimental

2.1. Chemicals and reagents

4-Nonylphenol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bisphenol A (purity of 99.9%) was purchased from Shanghai Reagent Factory (Shanghai, China). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany) and Ludu Chemical Reagent Factory (Shanghai, China), respectively. Milli-Q water (Millipore, Bedford, MA, USA) was used throughout. The other chemicals used were of analytical reagent grade.

Stock standard solutions of 0.50 mg/ml BPA and 0.60 mg/ml 4-NP were prepared in methanol, respectively. Standard working solutions of 0.01–50 μg/ml for BPA and 0.15–150.0 μg/ml for 4-NP were prepared by dilution of the stock standard solutions with methanol.

2.2. Chromatographic system and conditions

The HPLC system used was a HP1100 HPLC (Agilent, USA), consisting of a quaternary pump (G1311A), a fluorescence detector (Agilent, USA) set at an excitation wavelength of 227 nm and an emission wavelength of 313 nm. A 7725i injector (Rheodyne, USA) was used. Data collection and integration were accomplished using Hewlett-Packard Chemstation.

The analytical column was a Phenomenex ODS (250 mm × 4.6 mm I.D., 5 μm, USA). Mobile phase A was acetonitrile and mobile phase B was water. The system was run in a linear gradient: 0–6.0 min A 70%, B-30%, 6.0–6.2 min mobile phase A increased from 70% to 100%, held for 6.8 min, after 13 min A-70%, B-30% and stopped at 18 min. The chromatographic analysis was performed at 25 °C with flow-rate of 1.0 ml/min and injection volume of 20 μl.

2.3. Animals and tissue sample preparation

Male Wistar rats were used in the experiments (body weights of 180–250 g, obtained from Huaxi Experimental Animals Center, Sichuan University, China).The animals were kept in a room maintained at 24 ± 1 °C and housed three per cage. Twelve hours before BPA and 4-NP oral administration, the rats were fasted and provided only tap water.

BPA and 4-NP were dissolved in corn oil before administration. Rats were divided into three groups at random by body weight. Group 1 was administered a single dose of BPA (100 mg/kg, oral administration, $n = 72$), group 2 was administered a single dose of 4-NP (200 mg/kg, oral administration, $n = 72$), group 3 was administered a combination dose of BPA and 4-NP (100 mg/kg of BPA and 200 mg/kg of 4-NP, oral administration, $n = 72$). There are three subgroups in each group with 24 rats per subgroup. Each group includes eight time points with nine rats sacrificed at 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h and 96 h, respectively. This makes a total of $n = 216$ rats for three treatment groups. Considering that inter-variation between rats in terms of tissue size and weight, the same tissues were taken at random from three rats sacrificed per time point in each of the three subgroups and mixed to yield a sample for analysis. In addition, the control group was administered corn oil ($n = 9$). The rat blood was drawn and serum was prepared, the samples of the tissues (liver and testis) were stored at −20 °C until analysis.

2.4. Extraction of serum and tissue samples

2.4.1. Extraction of serum sample

Serum samples were extracted using the same procedure as described previously [16]. Hundred microlitres of 0.01 mol/l ammonium acetate buffer (pH 4.5) and 4 ml mixed solvent of n-hexane and diethyl ether (70:30, v/v) were added to 500 μl of serum. The samples were centrifuged and the organic layer was evaporated to dryness under gentle nitrogen flow. The samples were reconstituted with 100 μl of acetonitrile for analysis.

2.4.2. Extraction of tissue samples

The liver and testis samples of 0.50–1.00 g were accurately weighed. The samples were homogenized with 2.0 ml of 0.01 mol/l ammonium acetate buffer (pH 4.5) for 1 min at room temperature. 8.0 ml of methanol and 100 μl of 4.0 mol/l perchloric acid were added to the homogenate. The sample was agitated on a vortex mixer, sonicated for 10 min and centrifuged for 10 min at 12,000 rpm (Sorvall Instruments, Du Pont, USA).

The supernatant of 5.0 ml was taken to a glass tube, to which 0.01 mol/l of ammonium acetate buffer (pH 4.5) was added up to 20 ml and mixed to yield a solution. The C18 SPE cartridge (500 mg, 3 ml, BESEP, USA) was conditioned with 10 ml of methanol and equilibrated with 5 ml of water and 5 ml of 0.01 mol/l ammonium acetate buffer (pH 4.5) prior to use. Then the sample solution was loaded to the cartridge, washed with 5 ml of water (residual water was removed by placing the cartridge under vacuum for 30 s) and eluted with 4.0 ml of methanol at a low flow rate (1 ml/min). If the sorbent in the cartridge runs dry anytime before the sample-loading step into the cartridge, the consequence is low and variable recovery [17–20]. The eluting solution was evaporated to dryness with the rotary-vacuum evaporation apparatus (Yinyu, Gongyi, China) at 45 ± 1 °C and reconstituted in 100 μl of acetonitrile. The obtained samples were analyzed by HPLC.

2.5. Toxicokinetics and statistical analysis

The concentrations of BPA and 4-NP in serum, liver and testis were calculated from the corresponding calibration curves. Toxicokintic parameters were obtained with Practical Pharmacokinetic Program software [21] handling the test data of BPA and 4-NP, including the peak concentrations (C_{max}), concentration peak times (T_{max}), the area under the curve for concentrations versus time (AUC), the terminal elimination rate constant (K_{el}), the elimination half-time ($t_{1/2}$), the clearance (Cl_s) and the mean residence time (MRT). All data are presented as mean ± standard deviation ($\bar{X} \pm$ S.D.). The statistical significance of difference between mean values was assessed by Student's *t*-test with $p < 0.05$ (two-tailed) being considered significantly different.

3. Results and discussion

3.1. Optimization of chromatographic condition

In this study, the chromatographic conditions were carefully studied in order to find a suitable mobile phase with good resolution for BPA, 4-NP and the endogenous interference substances from rat serum and tissues. Mobile phases including acetonitrile (or methanol) and water with different eluting programs were tested. To begin with, various proportions of acetonitrile from 50% to 80% (v/v) as the mobile phase were tested. A good separation was obtained between BPA and the interfering substances in serum and tissue samples when a gradient elution program was chosen as follows: acetonitrile:water (70%:30%, v/v) was used within the first 6.0 min, then the proportion of acetonitrile was rapidly increased from 70% to 100% in 0.2 min and kept for 6.8 min to speed up the elution of 4-NP, then the mobile phase returned to acetonitrile:water (70%:30%) for 5 min and stopped. It was observed in our experiments that replacement of acetonitrile by methanol would result in poor peak shape and the fluctuating baseline. A satisfactory separation of BPA and 4-NP was achieved within 13 min with the retention times of 3.7 min and 11.0 min for BPA and 4-NP, respectively. The chromatograms are shown in Fig. 1.

3.2. Optimization of SPE

3.2.1. Investigation of BPA and 4-NP contamination in SPE cartridge

As the presence of BPA and/or 4-NP in plastic products is ubiquitous, such as polycarbonate and polyvinyl chloride products [11,22], it is possible that contamination might arise from the materials made of SPE cartridge themselves. Therefore, we tried to investigate if the SPE cartridge was contaminated with BPA and/or 4-NP prior to use.

The C18 SPE cartridges (500 mg, 3 ml, BESEP, USA) were used in the experiments. The migration test was conducted by soaking a SPE cartridge in 10 ml methanol for 30 min at room temperature. Then, the methanol was evaporated and dried with the rotary-vacuum evaporator at 45 ± 1 °C. The sample was reconstituted in 100 μl of acetonitrile and determined by HPLC. The experimental results indicated that the contents of BPA and 4-NP in the SPE cartridge were lower than their limits of detection (LOD) in the proposed method. Therefore, the C18 cartridge can be used in the solid-phase extraction.

3.2.2. Selection of the eluent volume in the solid-phase extraction

The volume of methanol as elution solvent in the solid-phase extraction was also examined. One gram of liver sample administered with the high concentration of analytes was accurately weighed and treated with the procedure of "extraction of tissue samples" (Section 2.4.2) described above. The analytes on a C18 cartridge was eluted in turn with different volume of methanol, namely 0.50 ml, 0.50 ml, 1.0 ml, 1.0 ml, 1.0 ml, and 1.0 ml collected and evaporated to dryness with nitrogen stream, respectively. The residues were reconstituted in 100 μl of acetonitrile for HPLC analysis. The results were shown in Table 1. The accumulative amount of the analytes desorbed from the SPE cartridge increased with the increase of the eluent volume. Methanol of 4.0 ml was chosen as the adequate volume for the elution in our experiments.

3.2.3. Extraction efficiency of BPA and 4-NP using SPE

The extraction efficiencies of the SPE procedure for BPA and 4-NP were calculated by comparing each absolute peak area of the mixed standard solution with or without the SPE procedure at three different concentration levels (repeated measurements for three times). The average extraction efficiencies ranged from 84.8% to 97.9%, as shown in Table 2. In serum and tissue samples, the extraction efficiencies of the SPE procedure for BPA and 4-NP were ranging from 78.6% to 95.0%.

Table 1
Incremental elution efficiencies of BPA and 4-NP obtained with different volumes of elution solvent (%)

Chemicals	Elution volume of methanol (ml)					
	0.50	0.50	1.0	1.0	1.0	1.0
BPA	75.2	24.4	0.29	0.06	0.04	0.01
4-NP	26.0	67.8	5.40	0.55	0.15	0.10

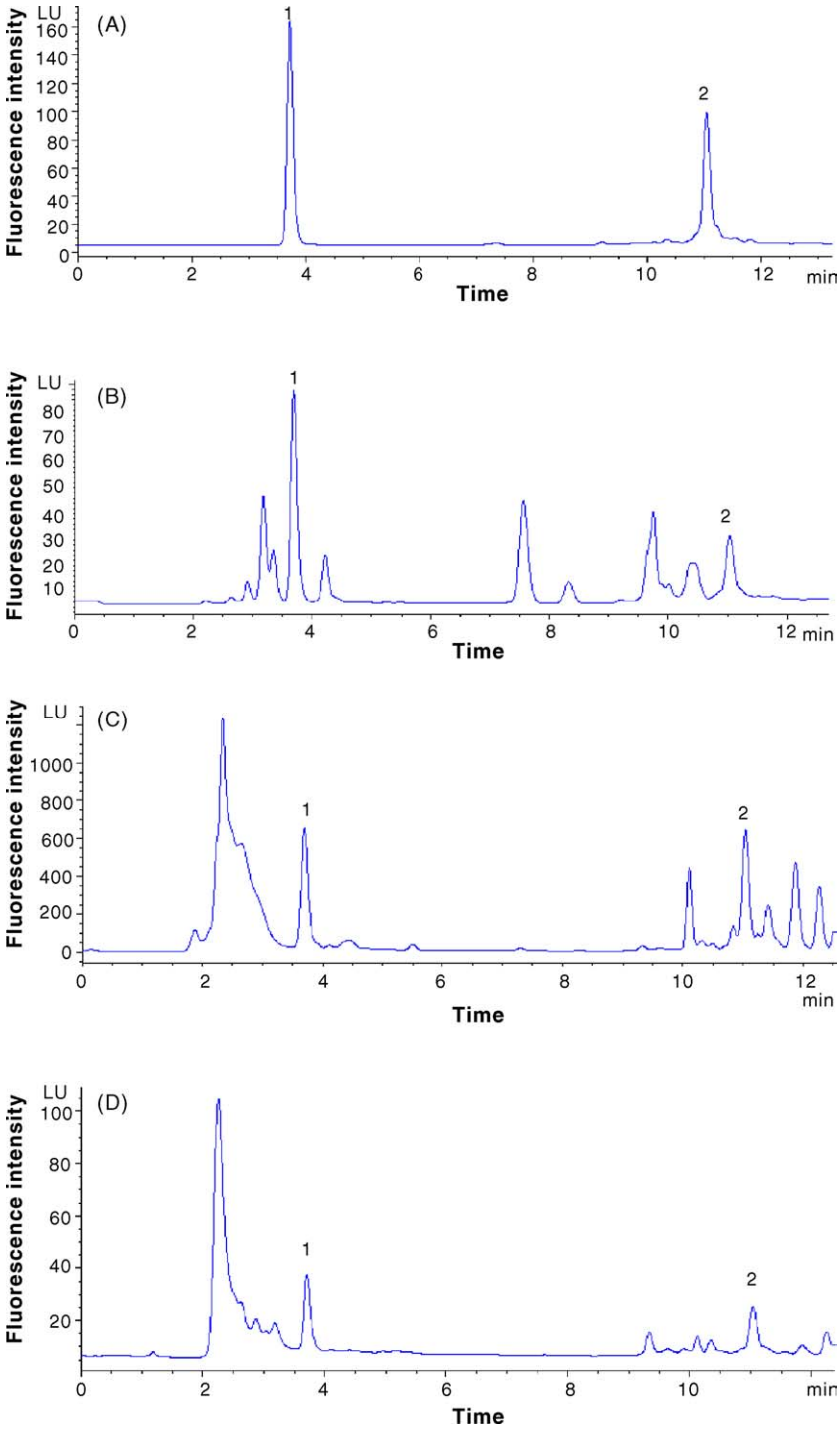

Fig. 1. Chromatograms of bisphenol A and 4-nonylphenol (1) Bisphenol A, (2) 4-nonylphenol (A) standard solution (BPA:5 μg/ml, 4-NP:7.5 μg/ml); (B) serum sample at 8 h following BPA and 4-NP co-administration; (C) liver sample at 8 h following BPA and 4-NP co-administration and (D) Testis sample at 8 h following BPA and 4-NP co-administration.

3.3. Method validation

3.3.1. Calibration curves and LOD_s

The calibration curves were prepared over the ranges of 0.01–50.0 μg/ml for BPA and 0.15–150.0 μg/ml for 4-NP. The mixed standard solutions of 20 μl were injected in the HPLC, respectively, and calibration curves were constructed by plotting the peak areas (Y) against the concentrations (X) for each the analyte. Excellent linear relationships were demonstrated between peak areas and the corresponding standard concentrations of BPA and 4-NP. The regression lines from the calibration runs were described by $Y = 191.11X + 21.58$ ($r = 0.9999$) for BPA and $Y = 99.15X + 34.12$ ($r = 0.9999$) for 4-NP. The limits of detection were 0.14 ng for BPA and 0.28 ng for 4-NP at a signal-to-noise

Table 2
Extraction efficiencies of BPA and 4-NP from aqueous standards using SPE ($n = 3$)

BPA		4-NP	
Added (μg/ml)	Average recovery (%)	Added (μg/ml)	Average recovery (%)
1.0	85.1	1.5	84.8
5.0	90.8	7.5	92.1
10.0	95.0	15.0	97.9

ratio of 3. The limits of detection for serum of 500 μl and tissue sample of 1.00 g were 2.8 ng/ml and 1.4 ng/g for BPA and 5.6 ng/ml and 2.8 ng/g for 4-NP, respectively.

3.3.2. Precisions and recoveries

The precision of the method was evaluated by replicated measurement of two levels of the mixed standard solution and the precision was calculated as the relative standard deviation (R.S.D.) within a single run (intra-assay) and between different assays (inter-assay). The intra-assay R.S.D. was better than 3.0% and inter-assay R.S.D. was 5.0–11.4%, as shown in Table 3.

Recoveries were assessed by analyzing spiked rat serum, liver and testis samples with known concentrations of BPA and 4-NP at three levels with repeated determinations for three times. The recoveries were 78.6–95.0% and 80.2–93.4% for BPA and 4-NP, respectively. The results were shown in Table 4.

3.4. Toxicokinetics of BPA and 4-NP in rat serum, liver and testis tissues

The HPLC method described above has been applied to the determination of BPA and 4-NP in rat serum and tissues and their toxicokinetic study. The toxicokinetic calculation was processed by carrying out with the Practical Pharmacokinetic Program-version 87 (edited by the Chinese Society of Mathematical Pharmacology, version 1.0).

Previous studies comparing the estrogenic activity of NP or BPA and their glucuronide metabolites indicated that only the parent compounds have estrogen-like activity [23–25]. Therefore, the toxicokinetics of "free" (unconjugated) BPA and 4-NP was investigated in rat serum, liver and testis in the present work.

Table 3
Intra- and inter-day precision for the analysis of BPA and 4-NP of standard solutions, rat serum and tissue samples

Compound	Concentration (μg/ml)	Spiked (μg/ml)	Precision, R.S.D.%	
			Intra-assay ($n = 7$)	Inter-assay ($n = 5$)
Standard solution				
BPA	0.05		3.0	11.4
BPA	1.0		1.3	9.2
4-NP	0.50		3.2	10.2
4-NP	5.0		0.9	5.0
Serum				
BPA		5.00	0.3	8.2
4-NP		7.50	0.3	5.2
Liver				
BPA		2.50	0.1	6.0
4-NP		3.75	1.4	7.4
Testis				
BPA		2.50	1.3	5.6
4-NP		3.75	0.3	5.6

Based on the distributions of BPA and 4-NP into the rat serum and selected endocrine-responsive tissues in our previous study [15], BPA and 4-NP were orally administered at a dose of 100 mg/kg and 200 mg/kg, respectively, individually or in combination in this study. The profiles of BPA concentrations in serum, liver and testis versus time in the absence and presence of 4-NP are shown in Fig. 2A and B and their corresponding toxicokinetic parameters are listed in Table 5. The profiles of 4-NP concentrations in serum, liver and testis tissues versus time in the absence and presence of BPA are shown in Fig. 3A and B and their corresponding toxicokinetic parameters are listed in Table 6.

As shown in Table 5, except C_{max} and MRT, the other parameters of BPA in testis were significantly altered by 4-NP co-administration ($p < 0.05$, $t > 2.776$), especially T_{max} and AUC remarkably increased from 2.30 ± 0.27 h and 5.76 ± 1.33 μg h/g in the absence of 4-NP to 8.39 ± 0.96 h and 23.0 ± 7.67 μg h/g on 4-NP co-administration, which suggested that the absorption of BPA in rat body became stronger in the presence of 4-NP. Significant decrease of K_{el} and CL_s and increase of $t_{1/2}$ in testis after simultaneous oral administration of BPA and

Table 4
Recoveries of spiked samples with the proposed analytical method ($n = 3$)

Samples	Bisphenol A				4-Nonylphenol			
	Original (μg/ml)	Added (μg/ml)	Found (μg/ml)	Recovery (%)	Original (μg/ml)	Added (μg/ml)	Found (μg/ml)	Recovery (%)
Serum	0.970	1.00	1.805	83.5	0.501	1.50	1.704	80.2
	0.970	5.00	5.370	88.0	0.501	7.50	6.830	84.4
	0.970	10.0	10.47	95.0	0.501	15.0	14.48	93.2
Liver	2.212	0.50	2.632	84.0	0.896	0.75	1.534	85.1
	2.212	2.50	4.382	86.8	0.896	3.75	3.945	81.3
	2.212	5.00	6.817	92.1	0.896	7.50	7.474	87.7
Testis	0.922	0.50	1.315	78.6	1.369	0.75	1.982	81.7
	0.904	2.50	3.304	96.0	1.335	3.75	4.554	85.8
	0.448	5.00	4.947	89.9	1.327	7.50	8.329	93.4

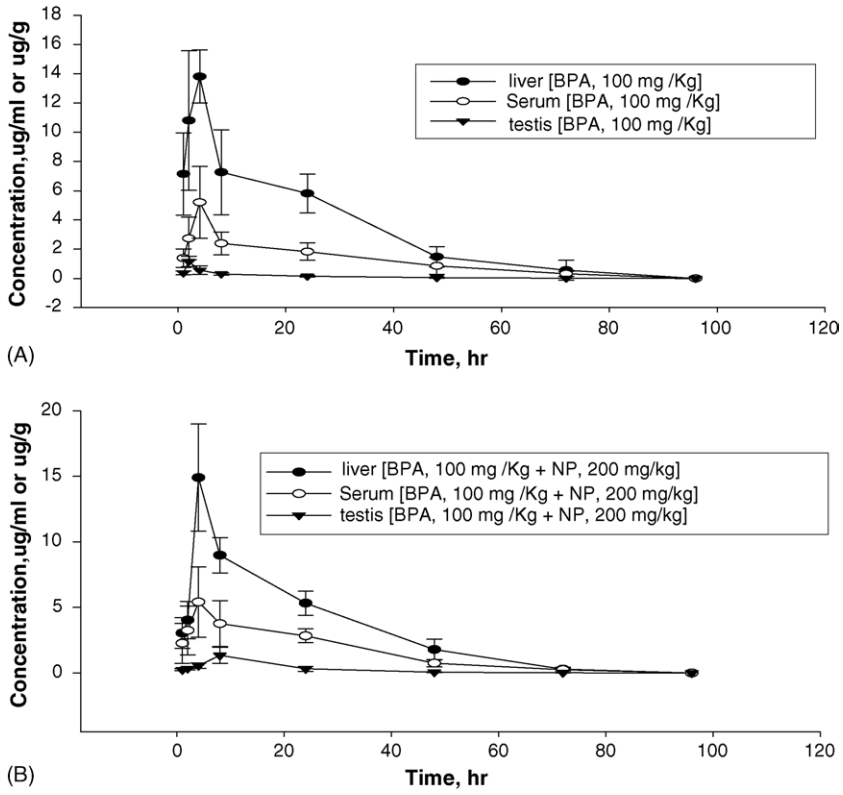

Fig. 2. Mean concentration of BPA in serum, liver and testis tissues after (A) individual oral administration of BPA (100 mg/kg) and (B) simultaneous oral administration of BPA (100 mg/kg) and 4-NP (200 mg/kg) ($n = 3$).

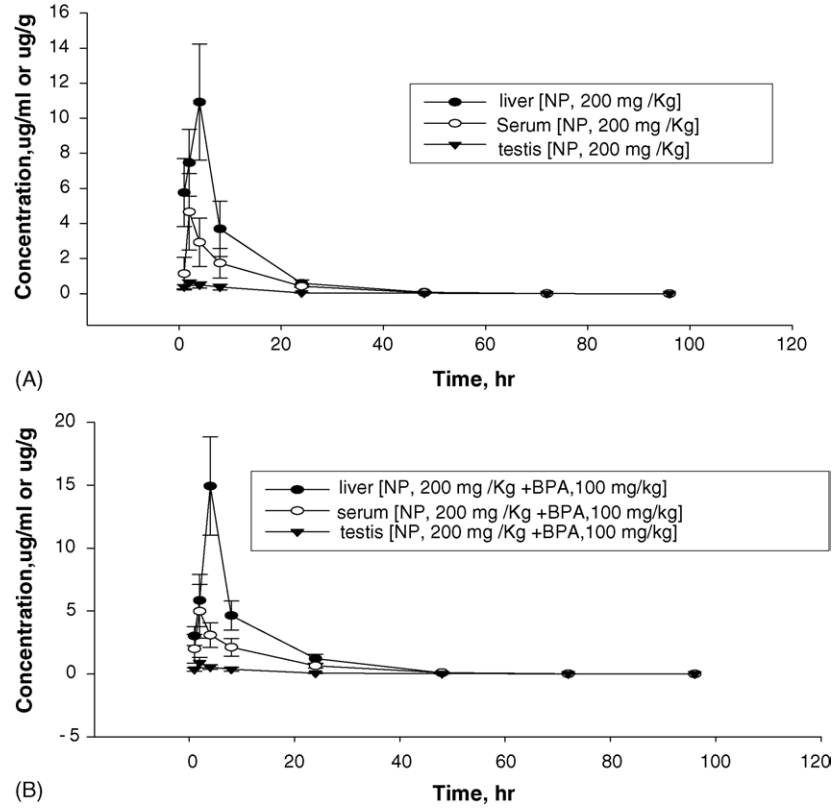

Fig. 3. Mean concentration of 4-NP in serum, liver and testis tissues after (A) individual oral administration of 4-NP (200 mg/kg) and (B) simultaneous oral administration of BPA (100 mg/kg) and 4-NP (200 mg/kg) ($n = 3$).

Table 5
Toxicokinetics of BPA (100 mg/kg) in serum, liver and testis after single oral administration to rats and in combination with NP (200 mg/kg) ($n = 3$)

	C_{max} (μg/ml or g)	T_{max} (h)	$t_{1/2}$ (h)	AUC (μg/g or ml) h	K_{el} (h^{-1})	MRT (h)	CLs (mg/kg/h/(μg/g or ml))
Serum							
I	3.98 ± 0.63	4.06 ± 1.47	16.47 ± 1.57	109.4 ± 15.6	0.043 ± 0.0057	24.4 ± 3.57	0.93 ± 0.139
II	4.64 ± 1.15	4.65 ± 0.19	18.38 ± 2.0	144.3 ± 29.4	0.037 ± 0.0025	20.4 ± 1.09	0.71 ± 0.162
Liver							
I	11.2 ± 1.18	3.15 ± 0.52	14.76 ± 1.93	285.8 ± 27.6	0.050 ± 0.0015	19.7 ± 1.43	0.35 ± 0.032
II	12.4 ± 1.03	5.72 ± 0.24* ($t = 7.77$, $p < 0.002$)	13.74 ± 0.3	298.9 ± 2.64	0.047 ± 0.0061	20.1 ± 0.93	0.33 ± 0.004
Testis							
I	0.85 ± 0.10	2.30 ± 0.27	2.32 ± 0.50	5.76 ± 1.33	0.31 ± 0.076	15.7 ± 2.17	17.9 ± 3.88
II	1.04 ± 0.29	8.39 ± 0.96* ($t = 10.5$, $p < 0.001$)	7.07 ± 0.74* ($t = 9.2$, $p < 0.001$)	23.0 ± 7.67* ($t = 3.84$, $p < 0.02$)	0.098 ± 0.108* ($t = 2.78$, $p < 0.05$))	14.7 ± 0.16	4.63 ± 1.31* ($t = 7.29$, $p < 0.002$

Data are expressed as mean ± S.D. (I) Individual oral administration of BPA(100 mg/kg) to rats. (II) Simultaneous oral administration of BPA (100 mg/kg) and NP (200 mg/kg) to rats. C_{max}: the peak concentrations; T_{max}: concentration peak times; $t_{1/2}$: the elimination half-life; AUC: the area under the curve for concentrations vs. time; K_{el}: the terminal elimination rate constant; MRT: the mean residence time; Cls: the clearance.
* $p < 0.05$, $t > 2.776$, significantly different from individual oral administration of BPA.

4-NP observed in our experiments may indicate that an obviously enhanced accumulation and slower elimination of BPA happened in testis during co-administration. The dose of 4-NP at 200 mg/kg has probably saturated the metabolism of BPA (given at 100 mg/kg) in the rats, thereby slowing down its elimination after co-administration with 4-NP [26]. On the other hand, except T_{max} in liver, the toxicokinetic parameters of BPA in serum and liver were not significantly changed after simultaneous oral administration of BPA and 4-NP compared with individual administration of BPA.

It can be seen in Table 6 that only MRT of 4-NP in liver was significantly altered by BPA co-administration, while the other parameters of 4-NP in serum, liver and testis were not remarkably altered by BPA co-administration. Compared with the toxicokinetics of BPA in Table 5, it seems to be a less influence of BPA co-administration on the toxicokinetics of 4-NP from Table 6.

As shown in Tables 5 and 6, the AUCs of BPA and 4-NP in liver have the highest values than those in serum and testis and at all time points the concentrations of BPA or/and 4-NP in serum and tissues were ranked: liver > serum > testis. These observations indicated that BPA and 4-NP introduced into rat body are mainly distributed in liver, next in serum. A small fraction of BPA or/and 4-NP can reach the testis after individual or simultaneous oral administration to rats in our experiments, which may also suggest that these compounds could pass through the testicular barrier due to their lipid-soluble properties [12,27] and small molecular mass. This was consistent with the suggestion of H. Miyakoda et al. about free BPA in testis from mature male rats after oral administration [12]. The elimination half-time ($t_{1/2}$) in serum for 4-NP in these rats after individual administration was similar to those observed for total 4-NP in male Sprague-Dawley rats after oral ingestion [13]. However, the elimination half-time ($t_{1/2}$) and T_{max} of 4-NP and BPA were getting longer in simultaneous oral administration of BPA and 4-NP at the doses given here, which may result from the saturation of their detoxification pathways.

From the previous studies [13,24,26,28–30], it is noticed that rats eliminate BPA (and 4-NP) less efficiently than humans. This is largely due to a larger fraction of conjugates excreted with bile followed by enterohepatic recirculation in rats. In humans, renal excretion is more important, and thus, also half-lives are shorter

Table 6
Toxicokinetics of NP (200 mg/kg) in serum, liver and testis after single oral administration to rats and in combination with BPA (100 mg/kg) ($n = 3$)

	C_{max} (μg/ml or g)	T_{max} (h)	$t_{1/2}$ (h)	AUC (μg/g or ml) h	K_{el} (h^{-1})	MRT (h)	CLs (mg/kg/h/(μg/g or ml))
Serum							
I	4.15 ± 1.60	1.77 ± 0.49	3.33 ± 1.08	30.76 ± 11.7	0.223 ± 0.0666	10.3 ± 0.89	7.06 ± 2.197
II	4.59 ± 1.26	2.10 ± 0.66	5.73 ± 2.38	39.97 ± 14.1	0.140 ± 0.0781	12.0 ± 0.66	5.38 ± 1.164
Liver							
I	8.93 ± 1.56	2.58 ± 1.64	3.37 ± 1.06	88.0 ± 12.8	0.221 ± 0.0777	7.93 ± 0.15	2.30 ± 0.308
II	11.7 ± 0.77	3.82 ± 0.39	3.76 ± 1.07	95.4 ± 19.6	0.197 ± 0.0668	10.1 ± 1.17* ($t = 3.19$, $p < 0.05$)	2.15 ± 0.398
Testis							
I	0.59 ± 0.10	2.67 ± 0.50	4.74 ± 2.39	6.65 ± 0.17	0.139 ± 0.0329	10.7 ± 0.07	30.1 ± 0.776
II	0.69 ± 0.14	2.76 ± 0.57	5.39 ± 1.26	7.25 ± 0.63	0.129 ± 0.046	11.3 ± 0.96	27.3 ± 2.608

Data are expressed as mean ± S.D. (I) Individual oral administration of NP (200 mg/kg) to rats. (II) Simultaneous oral administration of NP (200 mg/kg) and BPA(100 mg/kg) to rats. C_{max}: the peak concentrations; T_{max}: concentration peak times; $t_{1/2}$: the elimination half-life; AUC: the area under the curve for concentrations vs. time; K_{el}: the terminal elimination rate constant; MRT: the mean residence time; Cls: the clearance.
* $p < 0.05$, $t > 2.776$, significantly different from individual oral administration of 4-NP.

in humans than in rats. Moreover, known exposures of humans are much lower (about 10 μg/day for BPA and 7.5 μg/day for 4-NP [31,32]) than the experimental doses applied here. Therefore, further study on the toxicokinetic interactions of BPA and 4-NP at low doses is necessary to evaluate the effect on humans in the presence of low environmental dietary exposures to BPA and 4-NP.

4. Conclusion

A simple, sensitive and accurate SPE-extraction-HPLC method with direct fluorescence detection has been developed for simultaneous determination of BPA and 4-NP in rat serum, liver and testis tissues in this work. The proposed method will provide a useful way to investigate the environmental exposure levels of BPA and 4-NP in biologic samples and it has been successfully applied to the study of toxicokinetic interaction between the two environmental estrogenic compounds of BPA and 4-NP in rats. 4-NP significantly altered the toxicokinetics of BPA in rat testis under the experimental conditions investigated. Yet, it is highly unlikely that kinetics are affected at much lower doses, such as those encountered by humans from dietary exposure to BPA and 4-NP. Nonetheless, combinations require further attention with respect to toxicokinetic interactions.

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