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Development of sensitive high-performance liquid chromatography with fluorescence detection using 4-(4,5-diphenyl-1*H*-imidazol-2-yl)-benzoyl chloride as a labeling reagent for determination of bisphenol A in plasma samples

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Abstract

A sensitive HPLC method for determination of bisphenol A (BPA) in plasma samples using 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) as a fluorescence labeling reagent was developed. The fluorescence labeling reaction was completed within 10 min at room temperature. DIB-Cl reacts with the phenolic hydroxyl group of BPA in the presence of triethylamine (TEA). The DIB-Cl derivative of BPA (DIB-BPA) was separated within 30 min with an ODS column using acetonitrile–water (90:10, v/v) as the isocratic eluent. Calibration graphs were linear over the range of 1.0–100 ng/ml (r=0.999). The detection limit of DIB-BPA was 0.05 ng/ml (2.5 pg) at a signal-to-noise ratio of 3. The relative standard deviations (RSDs) of the method for between-run were 1.0–5.0%. The analytical recoveries of known amounts (1.0 and 100 ng/ml) of BPA-spiked rabbit plasma were around 95%.

Keywords: Bisphenol A; 4-(4,5-Diphenyl-1*H*-imidazol-2-yl)benzoyl chloride

1. Introduction

Bisphenol A (BPA) has been widely used as a raw material for polycarbonate and epoxy resin to produce various products. As a result, BPA is present in many household plastic products and in food packaging materials. Therefore, its leaching from polycarbonate baby bottles [1], epoxy can coatings [2,3], canned foods or drinks [4–6], several types of dental sealants [7–9] and plastic wastes [10] has been reported. Recently, it has been found that BPA

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can affect animal physiology by mimicking the effects of hormones.

The estrogenicity of BPA has been demonstrated in a number of in vitro and in vivo assays. It is clear from in vitro assays including E-SCREEN assay [11,12], estrogen-dependent reporter-gene expression response [13], estrogen receptor binding assay [14] and two-hybrid assay [15] that BPA has estrogen activity. Nagel et al. [16] reported that administration of low oral doses of BPA to pregnant female mice on gestation days produced statistically significant increases in the weights of the prostate and a decrease in epididymis weights in their male offspring. In contrast, there is a study that disputes the low dose effects of BPA [17]. Recently, it was also shown that mouse fetuses exposed to BPA at a dose within the range typical of the environmental exposure of humans alters the postnatal growth rate and brings on early puberty in these mice [18]. These results have advocates and skeptics, however, the ultimate resolution of the debate will rest on results of ongoing assessments of BPA. Therefore, analysts must develop and validate methods for determination of trace amounts of BPA in various samples.

The aim of this study is to develop a method for determination of trace amounts of BPA. BPA in the air has been determined by using GC–MS [19]. del Olmo et al. [20] and González-Casado et al. [21] described methods for determining trace levels of BPA in aquatic samples by using GC–MS. Also, methods for determination of BPA by using high-performance liquid chromatography (HPLC) have been reported. These methods employ HPLC with various detectors including UV [22], fluorescence detection [23], electrochemical detection [24], and mass spectrometry [25]. Moreover, methods using ELISA [26] and spectrofluorimetry [27,28] have also been developed. Recently, Sun et al. [29] reported a selective and sensitive method using a derivatization reagent for chemiluminescence detection. Sensitive analyses for primary amines [30] and phenolic compounds [31,32] using 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) as a fluorescence derivatization reagent have also been reported.

We applied a fluorescence detection method with DIB-Cl to determination of trace levels of BPA. Only a few selective and sensitive methods for determination of trace amounts of BPA in biological samples have been reported [24,25]. In these reports, free-BPA was observed to be present in human blood samples at very low levels. This result is supported by a study showing that bioavailability of BPA was low in female DA/Han rats [33], and a study showing that BPA was metabolized by glucuronidation [34]. To confirm the presence of BPA at very low levels, a highly sensitive and selective method for determination of BPA is necessary. Therefore, we aimed to develop such a method and apply it to determining trace levels of BPA in plasma samples.

2. Experimental

2.1. Reagents and samples

BPA standard of environmental analytical grade was purchased from Wako Pure Chemicals Industries (Tokyo, Japan). Acetonitrile, methanol and triethylamine (TEA) were obtained from Wako Pure Chemicals Industries. DIB-Cl was synthesized in our laboratory according to the method reported by Nakashima et al. [31]. Water purified with Milli-Q water purification system (Millipore, USA) was used. Recently, we reported that a trace amount of BPA was detected in Milli-Q-purified water which is normally used in experiments [24]. In this study, considerable amounts of BPA were found to leach into laboratory water from plastic products used. Accordingly, we used pure water purified with a Milli-Q gradient A10 Elix with EDS polisher system water purification. The EDS polisher system comprises an activated carbon cartridge for Milli-Q gradient A10 Elix, and it was developed by Millipore Japan for analysis of plastic compounds such as phthalate, BPA and VOCs. Therefore, this water was useful for analysis of trace levels of BPA. Plasma samples were obtained from a Japanese white male rabbit (4.5 kg).

2.2. Equipment

The HPLC system consisted of a Shimadzu LC10Advp pump and a Shimadzu RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). A data processor used for recording was a C-R6A (Shimadzu, Kyoto, Japan). The column was a YMC-

Pack Pro C_{18} (4.6×250 mm I.D., 5 μm) by YMC Japan. Solid phase extractions for pretreatment to remove interfering substances in plasma samples were Sep-Pak Vac (1 ml, 50 mg) and Oasis HLB (3 ml, 60 mg) by Waters, as well as a Bond Elut SCX (1 ml, 100 mg) by Varian.

2.3. HPLC condition

The DIB-BPA standard was separated with acetonitrile–water–methanol (60:6:34, v/v) as a mobile phase at a flow-rate of 1.0 ml/min. The detection wavelength was an excitation of 340 nm and an emission of 470 nm. The amount of injection was 5.0 μl. DIB-BPA in plasma samples was separated by acetonitrile–water (9:1, v/v) as a mobile phase in the following manner under the conditions described above.

2.4. Derivatization reaction of BPA by DIB-Cl

A stock solution of 100 μg/ml of BPA was prepared in acetonitrile so that it would have the required concentration. Then, 200 μl of BPA standard solution or a sample was added to 200 μl of 15 m*M* DIB-Cl in acetonitrile containing 1.5% TEA; the mixture was stirred and allowed to stand for 10 mm at room temperature. Subsequently, 400 μl of a mobile phase solution was added to the mixture and the resultant mixture was stirred and allowed to stand for 30 min at room temperature. The resultant mixture solution was injected into HPLC.

2.5. Recovery test

One milliliter of a plasma sample was added to 1.0 ml of acetonitrile and 1.0 ml of 1 *M* hydrochloric acid, and the mixture was centrifuged at 3000 rpm for 10 min. Then, the supernatant was fractioned, to which 7.0 ml water was added, and solid-phase extraction (Oasis HLB) was performed. The solid-phase had been conditioned in advance by 15.0 ml methanol and 3.0 ml water, to which samples were added. The resultant mixture was washed with 3.0 ml water–methanol (4:1, v/v), and eluted by 3.0 ml methanol. The obtained solution was evaporated to dryness and then reconstituted in 200 μl acetonitrile. The solution was fluorescence derivatized according to the method described above. Then, in order to remove the effects of impurities derived from plasma, particularly amines and estradiol, the obtained solution was washed with 500 μl acetonitrile–water (2:1, v/v) using a Sep-Pak C_{18} column, and then eluted with acetonitrile (500 μl). Subsequently, the resultant derivative was cleaned up by washing consecutively with 3.0 ml water, 3.0 ml methanol, and 500 μl acetonitrile solution containing 1.5% TEA using a Varian SCX column, and then eluted with 1.0 ml acetonitrile solution containing 5% TEA. The obtained sample solution was subjected to HPLC.

3. Results and discussion

3.1. Review of the derivatization conditions

Sensitive analyses of primary amines and phenolic compounds using DIB-Cl as a fluorescence derivatization reagent have been reported. We used DIB-Cl for a sensitive analysis of BPA. Since BPA has a phenolic hydroxyl group to be labeled, the reaction scheme shown in Fig. 1 is predicted. To optimize the reaction, we studied the concentration of DIB-Cl, the concentration of TEA, the volume of DIB-Ci solution, and the reaction time.

3.1.1. Optimization of the DIB-Cl concentration

The concentration of DIB-Cl as a derivatization reagent was studied. The effect of the blank peak derived from a derivatization reagent cannot be ignored when the sensitivity is high. Thus, use of DIB-Cl having a minimum concentration is desirable. As shown in Fig. 2, the relative fluorescence intensity increased with an increasing concentration of DIB-Cl up to 15 m*M* and leveled off at 15 m*M* or

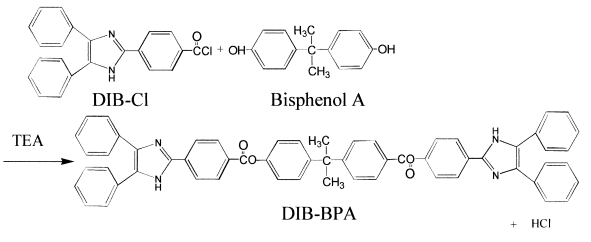

Fig. 1. The reaction scheme of BPA with DIB-Cl.

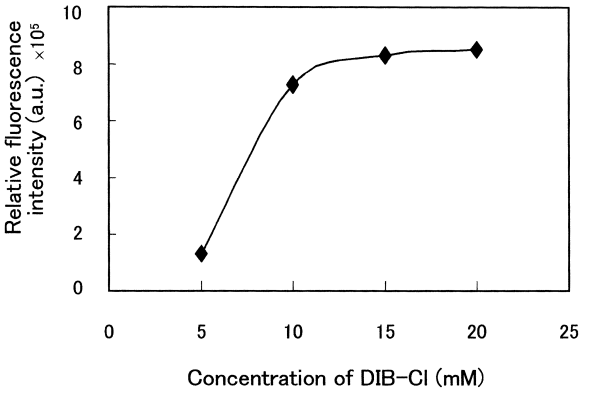

Fig. 2. Effect of DIB-Cl concentration on relative fluorescence intensity. Sample: 50 ng/ml BPA; TEA concentration: 1.5%; HPLC conditions: $CH_8CN–H_2O–CH_3OH$ (60:6:34, v/v); fluorescence detection: Ex.=340 nm, Em.=470 nm.

higher. Accordingly, the optimal concentration of DIB-Cl for determination of BPA was 15 m*M*.

3.1.2. Optimization of the TEA concentration

TEA has been known to serve as a catalyst promoting a reaction between DIB-Cl and a phenolic compound as well as to increase solubility. By use of 15 m*M* DIB-Cl, we studied the effects of the concentration of TEA. As shown in Fig. 3, the relative fluorescence intensity was stable when the concentration of TEA was 1.0% or higher. Accordingly, the optimal concentration of TEA for determination of BPA was 1.5%.

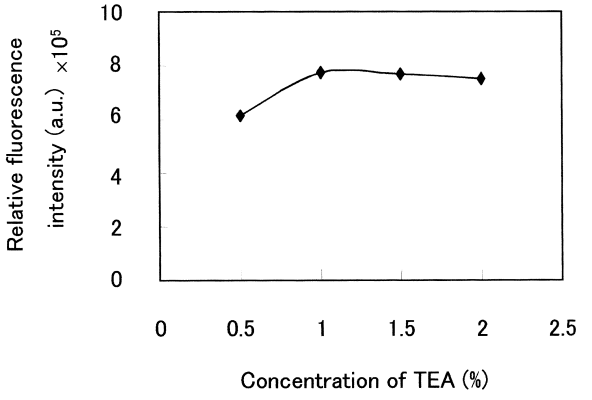

Fig. 3. Effect of TEA concentration on relative fluorescence intensity. Sample: 50 ng/ml BPA; DIB-Cl concentration: 15 m*M*; HPLC conditions: $CH_3CN–H_2O–CH_3OH$ (60:6:34, v/v); fluorescence detection: Ex.=340 nm, Em.=470 nm.

3.1.3. Optimization of the DIB-Cl volume

As shown in Fig. 1, the relationship between the volume of BPA and the volume of DIB-Cl, which was the fluorescence derivatization reagent, was studied. It is desirable to minimize the volume of DIB-Cl to suppress background.

As shown in Fig. 4, the relative fluorescence intensity increased with an increasing volume of DIB-Cl, and reached a peak around 200 μl. Over 200 μl of DIB-Cl, the relative fluorescence intensity was not expected to improve drastically and background may undesirably increase owing to an excess of DIB-Cl. Therefore, the optimal volume of DIB-Cl for determination of BPA was 200 μl.

3.1.4. Optimization of the reaction time

The reaction temperature was found to have no effect on the derivatization reagent. Thus, this study was conducted at room temperature. The BPA and DIB-Cl were reacted and allowed to stand for 10 min at room temperature. Then, the mobile phase was added to the mixture and the effect of the reaction time on the relative fluorescence intensity was examined. The result is shown in Fig. 5. The relative fluorescence intensity increased until 30 min after the reaction started and then decreased. Thus, the reaction time chosen for determination of BPA was 30 min when the relative fluorescence intensity peaked.

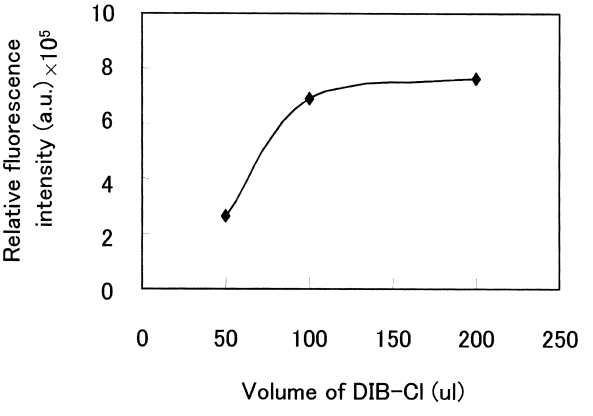

Fig. 4. Effect of DIB-Cl volume on relative fluorescence intensity. Sample: 50 ng/ml BPA; DIB-Cl concentration: 15 m*M*; TEA concentration: 1.5%; HPLC conditions: $CH_8CN–H_2O–CH_3OH$ (60:6:34, v/v); fluorescence detection, Ex.=340 nm, Em.=470 nm.

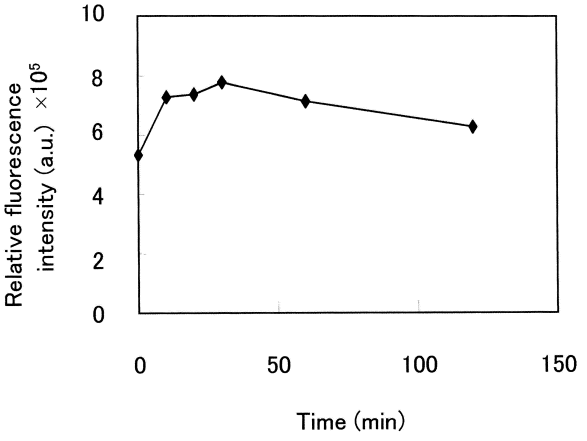

Fig. 5. Effect of reaction time on relative fluorescence intensity. Sample: 50 ng/ml BPA; DIB-Cl concentration: 15 m*M*; TEA concentration: 1.5%; HPLC conditions: $CH_8CN–H_2O–CH_3OH$ (60:6:34, v/v); fluorescence detection, Ex.=340 nm, Em.=470 nm.

The relative fluorescence intensity remained stable for a few hours before it started decreasing.

3.2. Optimization of the separation conditions

By using standard solutions, three types of catalysts that serve as a mobile phase were studied. The catalysts contained acetonitrile, water, and methanol in different ratios. When acetonitrile–water–methanol (70:5:25, v/v) was used, the retention time of DIB-BPA was 13.6 min, but separation from the blank peak was insufficient. When acetonitrile–water–methanol (70:7:23, v/v) was used, no effect of the blank peak was observed, but the retention time was as long as 18.4 min. When acetonitrile–water–methanol (60:6:34, v/v) was used, a good separation was achieved, but DIB-BPA in plasma samples was not sufficiently separated. When clean-up was performed in order to remove interfering substances by use of three types of solid-phase extraction columns and acetonitrile–water (9:1, v/v) as the mobile phase, a DIB-BPA derivative was sufficiently separated as shown in Fig. 6.

3.3. Analysis of characteristics and recovery tests

Detection characteristics of the proposed method under the optimal conditions will be described. The

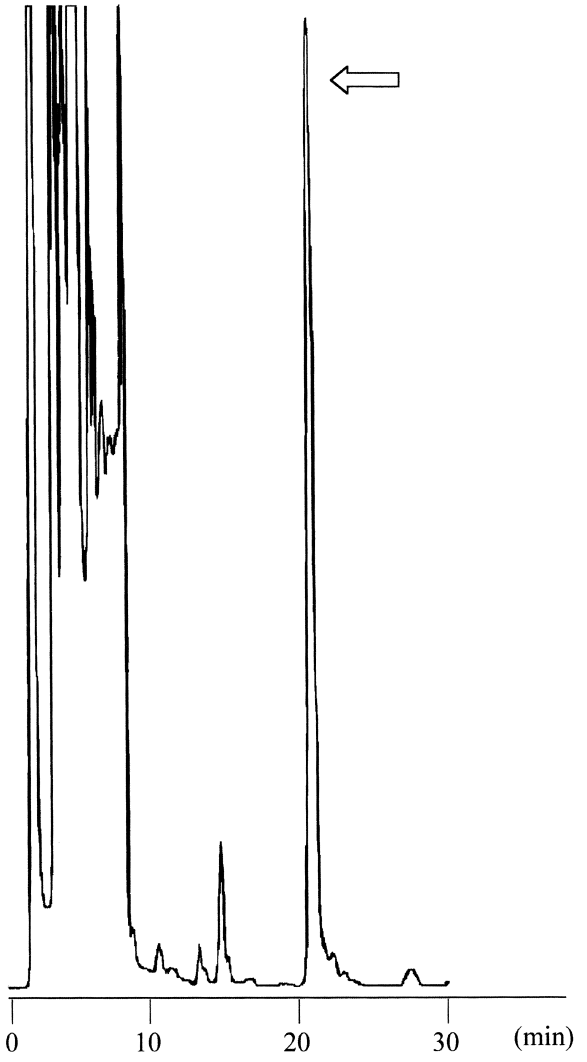

Fig. 6. Chromatogram of DIB-BPA derivative in rabbit plasma (100 ng/ml).

detection limit was calculated according to the ratio of the signal peak to the noise peak (S/N). The detection limit of BPA was 0.05 ng/ml (0.25 pg) ($S/N=3$). The labeling increased the fluorescence intensity of BPA by a factor of approximately 400. When the concentrations of BPA within the range of 0.1–100 ng/ml were measured, the calibration curve of the DIB-BPA standard showed a good linear relationship between the peak area and the peak height, with coefficients of correlation being at least $r=0.999$. The reproducibility at two concentrations,

Table 1
Recovery of DIB-BPA derivative from rabbit plasma ($n=4$)

Concentration (ng/ml)	Recovery (%)	RSD (%)
1	94.8	8.2
100	95.2	5.8

10 and 50 ng/ml, were studied by performing repeated analyses (five times). The precision of the analysis (RSD, %) was around 5.0% and the RSD of the retention time was 1.0% or less, both being found to be satisfactory. It was possible to separate and determine BPA with good precision by combining reversed-phase chromatography with highly selective and sensitive detection using DIB-Cl. The method yields a highly precise determination of standards and might be applied to detection of trace amounts of BPA in blood samples.

Table 1 shows the results of the recovery tests. When 1.0 or 100 ng/ml of BPA was added to rabbit plasma samples, the recoveries of BPA were 94.8 and 95.2%, respectively, and the RSDs were 8.2 and 5.8%, respectively. The limit of detection for measurement of BPA in plasma samples was 1.0 ng/ml, which was obtained by setting the ratio of the signal peak to the background noise of the control sample to be 10. We analyzed some rabbit plasma samples using the proposed method. The amount of BPA in plasma of a laboratory rabbit was below the limit of quantitation.

For various studies on the effect of low levels of BPA in vivo, it is necessary to measure the levels of free or metabolic BPA in biological samples of laboratory animals. Trace amounts of BPA have been demonstrated to stimulate estrogenic activity. Thus, it is necessary to develop a more sensitive and simpler analytical method for measuring BPA or BPA metabolites for this biomarker than any previously published method. In this study, we thus developed such a method and applied it to determination of trace levels of BPA in plasma.

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