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The Utility of Sonogashira Coupling Reaction for the Derivatization of Aryl Halides with Fluorescent Alkyne

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Aryl halides are a very important category of compounds that include many vital drugs and key industrial additives, such as clofibrate and bromobenzene, respectively. Due to their importance, our research group previously developed a novel fluorescence labeling approach for their analysis using a fluorescent aryl boronic acid as a reagent, based on the Suzuki coupling reaction. This coupling reaction was successfully applied for the determination of aryl halides in biological fluids; however, there was a limitation of low reactivity towards *ortho*-substituted aryl halides. In the present study, a novel fluorescence derivatization approach for aryl halides was developed using, 2-(4-ethynylphenyl)-4,5-diphenyl-1*H*-imidazole (DIB-ET) as a fluorescent alkyne reagent, based on the Sonogashira coupling reaction. DIB-ET reacted with aryl bromides in the presence of palladium and copper as catalysts, yielding fluorescent derivatives that could be subsequently determined by an HPLC system with fluorescence detection. The detection limits ($S/N = 3$) for aryl bromides were in the range of 14 – 23 nM, which is 3.5 – 18-times more sensitive than our previously developed approach, Suzuki coupling derivatization. Moreover, in contrast to the previous technique, the reactivity of DIB-ET to *ortho*-substituted aryl bromides was almost equivalent to that of the *para*-substituted aryl bromides. Hence, by using this newly developed approach we could label the aryl halides with more sensitivity and reactivity. Finally, the proposed method was successfully applied for the selective determination of aryl bromides in human serum with good recovery (84.6 – 107%), which proves the ability of the developed method to determine occupational exposure to aryl halides.

Keywords Fluorescence derivatization, Sonogashira coupling reaction, *ortho*-substituted aryl halide, fluorescent alkyne

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Introduction

Fluorescence derivatization is a useful technique for the sensitive HPLC analysis of non-fluorescent or weakly fluorescent compounds. A large number of reagents have been developed for the fluorescence derivatization of various functional groups such as amines, phenols, thiols, aldehydes and carbonyl groups.[1–7] However, conventional fluorescence derivatization reagents cannot be applied to many important compounds that do not possess such typical functional groups. An important example of such compounds is aryl halides. They are used as industrial additives and they are included in the structure of many pharmaceuticals.[8] This encouraged our research group to previously develop a fluorescence derivatization approach for aryl halides based on the Suzuki coupling reaction, which is a palladium catalyzed cross-coupling reaction between aryl halides and aryl boronic acid (Fig. 1a).[8] In this technique, we used fluorescent aryl boronic acid, 4-(4,5-diphenyl-1*H*-imidazole-2-yl)phenylboronic acid (DPA), as a derivatization reagent. DPA can selectively react with aryl halides to give fluorescent derivatives, even in the presence of numerous biological components. Until now, we successfully applied DPA for the monitoring of various aryl halides drugs including; clofibrate,[8] haloperidol[9] and hydroxyzine,[10] in biological fluids. However, this fluorescence derivatization approach suffered from low reactivity towards *ortho*-substituted aryl halides. This is attributed to the steric hindrance effect of *ortho*-substitution.[8]

In order to overcome this problem, we attempted to develop a novel fluorescence derivatization approach based on the Sonogashira coupling reaction which could improve the reactivity to *ortho*-substituted aryl halides. Sonogashira coupling is a palladium and copper-catalyzed carbon–carbon bond-forming reaction between aryl halides and terminal alkynes.[11] In the present study, we used the fluorescent alkyne, 2-(4-ethynylphenyl)-4,5-diphenyl-1*H*-imidazole (DIB-ET),[12] as a derivatization reagent for aryl halides (Fig. 1b). We expect that the formation of diphenyl acetylene by the reaction of DIB-ET with aryl halides should not be hindered in the presence of *ortho*-substituted functional group compared with the formation of biphenyl by the reaction of DPA and aryl halides.[13–16]

In this work, we firstly evaluated the usefulness of the proposed derivatization technique with DIB-ET for the HPLC analysis of aryl halides using aryl bromides as model analytes. Subsequently, we investigated the reactivity of DIB-ET towards *ortho*-, *meta*- and *para*-substituted aryl bromides; the results were compared with those of the previous derivatization technique that used DPA. Furthermore, the proposed method was applied for the determination of aryl bromides in spiked human serum samples to demonstrate the practicability in biological fluids. It is known that aryl bromides, such as

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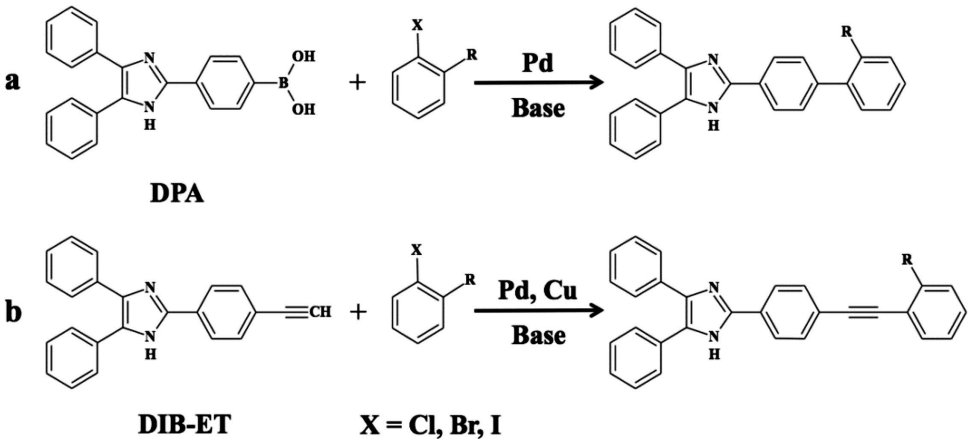

Fig. 1 Fluorescence derivatization of aryl halide by (a) the reaction with DPA based on the Suzuki coupling reaction and (b) the reaction with DIB-ET based on the Sonogashira coupling reaction (proposed method).

bromobenzene, are used as motor oil additives and as fire retardants.[17,18] However, it has been reported that aryl bromides exhibit certain toxicity such as hepatotoxicity and nephrotoxicity.[19-21] Therefore, their determination in serum should be necessary to investigate occupational exposure levels to toxic aryl bromides.

Experimental

Material and reagents

DIB-ET was synthesized in our laboratory according to the previously reported method by our research group.[12] Bromobenzene, *o*-bromoanisole, *p*-bromoanisole, *o*-bromotoluene, *p*-bromotoluene, *p*-chloroanisole, *p*-chlorotoluene, *N*,*N*-diisopropylethylamine (DIPEA), iodobenzene, *p*-iodoanisole and *p*-iodotoluene were purchased from Tokyo Chemical Industries (Tokyo). Chrolobenzene, tris{tris[3,5-bis(trifluoromethyl)phenyl]phosphine}palladium (super stable Pd catalyst), 1,4-dioxane, and *o*-iodoanisole were from Wako (Tokyo). *m*-Bromoanisole, copper (I) iodide (CuI) and acetonitrile were from Kanto Chemical (Tokyo). *m*-Bromotoluene was purchased from Sigma (St. Louis, MO). All of the used chemicals were of the highest purity and quality available, while the purities of the tested aryl halide were 98%, excluding bromobenzene (99%) and *p*-bromoanisole (97%).

The synthesis and characterization of the aryl halides authentic derivatives of DIB-ET are described in Supporting Information.

Aryl halides standard solutions, DIB-ET (3.0 mM), super stable Pd (2.5 mM), CuI (0.5 mM) and DIPEA (50 mM) reagent solutions were prepared in dioxane.

Fluorescence derivatization procedure for aryl halides

To a 50-µL dioxane solution of aryl halides, 50 µL aliquots of each of DIB-ET (3.0 mM), super stable Pd (2.5 mM), CuI (0.5 mM) and DIPEA (50 mM), were successively added and mixed in an amber-colored screw-capped vial. After purging with N_2 (5.0 mL/s) for 7 s, the reaction mixture was heated at 100°C for 40 min. After filtration through an Advantec (Tokyo) 0.45-µm PTFE membrane filter, 20 µL aliquot was injected into the HPLC system.

Assay procedure for aryl bromides in human serum

Human serum samples were spiked with aryl bromides (*o*-bromoanisole, bromobenzene, *o*-bromotoluene) with a final concentration of 50 µM. To a 150-µL portion of serum samples, 150 µL of ethyl acetate was added and vortex-mixed for 60 s. After centrifugation at 5000*g* for 10 min, 50 µL of the organic layer was taken and subjected to the derivatization, as described above.

HPLC system and conditions

The HPLC system consisted of a pump LC-6A (Shimadzu, Kyoto, Japan), a Shimadzu RF-550 fluorescence detector, a 7125 injector with a 20-µL loop (Rheodyne, Cotati, CA), and a chromate-recorder (SIC, Tokyo). Chromatographic separation was performed on a Cosmosil 5C18MS-II (250 × 4.6 mm, i.d., Nakalai Tesque, Kyoto) *via* isocratic elution with a mobile phase consisting of acetonitrile and water with a ratio of 7:3, respectively, at a flow rate of 1.0 mL/min. The excitation and emission wavelengths were set at 330 and 430 nm, respectively.

Results and Discussion

Optimization of the reaction between DIB-ET and aryl halides

In order to obtain higher reactivity, the derivatization reaction conditions were optimized using a standard solution of aryl bromides. The concentration of DIB-ET was investigated over a range of 0.5 – 10 mM, and the maximum peak heights of aryl bromides were obtained at a concentration of 3 mM (Fig. 2a). The concentration of super stable Pd was investigated over a range of 1 – 5 mM. The maximum and constant peak height of aryl bromides were obtained at more than 2.0 mM (Fig. 2b). 2.5 mM of super stable Pd was selected. In the Sonogashira coupling, CuI catalyzes the transmetalation of the alkynyl group to the Pd catalyst by the formation of a copper acetylide. The concentration of CuI was investigated over a range of 0.05 – 1 mM, and the maximum peak heights of aryl bromides were obtained at a concentration of 0.5 mM (Fig. S1, Supporting Information). Different kinds of bases including; DIPEA, trimethylamine (TEA), potassium fluoride (KF), potassium carbonate (K_2CO_3) and sodium carbonate (Na_2CO_3) were examined. Among the tested bases, DIPEA yielded the highest peak area for the targeted aryl bromides (Fig. S2, Supporting

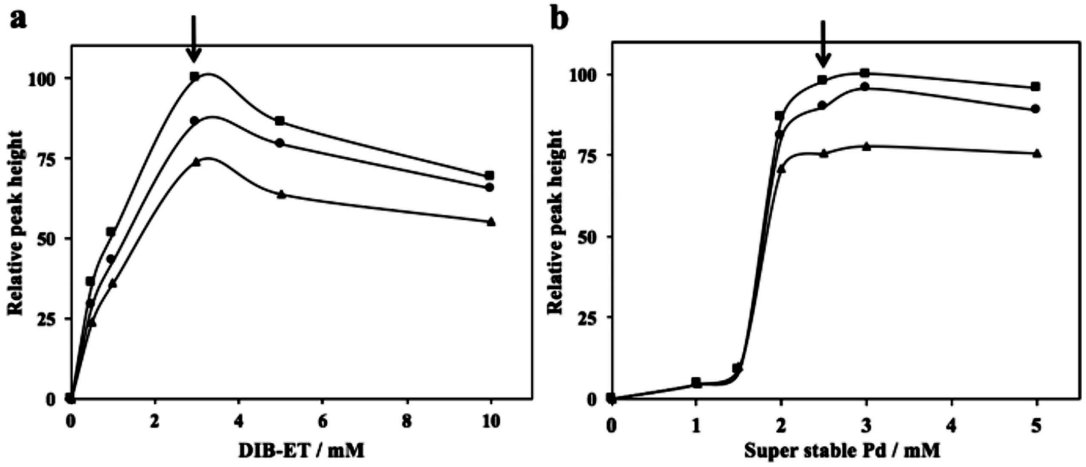

Fig. 2 Effect of the reagent concentrations and reaction conditions on the peak heights of aryl bromides. Where (a) effect of DIB-ET concentration, (b) effect of super stable Pd concentration. Compounds: ■, *p*-bromoanisole; ●, bromobenzene; ▲, *p*-bromotoluene and sample concentration: 50 μM for all compounds.

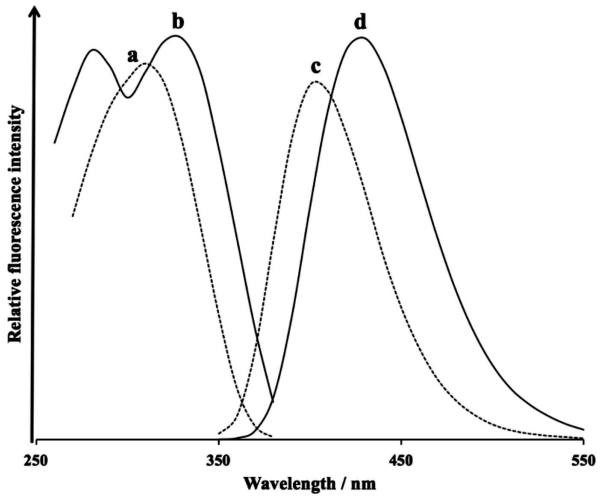

Fig. 3 Fluorescence spectra of the 0.5 μM DIB-ET and 0.5 μM bromobenzene derivative with DIB-ET. Excitation spectra of (a, dashed line) DIB-ET with emission at 400 nm and (b, solid line) bromobenzene derivative with emission at 430 nm. Emission spectra of (c, dashed line) DIB-ET with excitation at 310 nm and (d, solid line) bromobenzene derivative with excitation at 330 nm.

Information). Then, the concentration of DIPEA was investigated over a range of 2.5 – 100 and 50 mM of DIPEA was selected because it gave the maximum and constant peak heights (Fig. S3, Supporting Information). The effects of the reaction temperature and time were studied, and the optimum conditions were heating at 100°C for 40 min, as shown in Figs. S4 and S5 (Supporting Information), respectively. Figures S1 to S5 are provided in supplementary materials.

Fluorimetric study and chromatographic analysis of the formed aryl halides derivatives of DIB-ET

The fluorescence spectra of DIB-ET and the authentic derivative of aryl halides were measured with a Shimadzu RF-1500 fluorescence spectrometer. Figure 3 shows the fluorescence spectra of 0.5 μM DIB-ET (Figs. 3a and 3c) and 0.5 μM authentic bromobenzene derivative of DIB-ET (Figs. 3b and 3d). The fluorescence intensity of the bromobenzene derivative was 1.1-times higher than that of DIB-ET. Additionally, the maximum excitation and emission wavelengths of bromobenzene derivative (330/430 nm) were significantly longer than those of DIB-ET (310/400 nm). Therefore, the excitation and emission wavelengths of the fluorescence detector were set at 330 and 430 nm, respectively.

The chromatographic separation and analysis of aryl halides was carried out using the isocratic elution conditions mentioned previously. Figures 4a – 4c show chromatograms of the reagent blank, the reaction mixture of aryl bromides with DIB-ET and the synthesized authentic fluorescent derivatives, respectively. The peaks of the products of bromobenzene, *p*-bromoanisole and *p*-bromotoluene in the reaction mixture were detected at 21, 23 and 32 min, respectively. The retention times of these peaks were identical to those of the authentic derivatives. Therefore, it was confirmed that aryl bromides could be converted to fluorescent derivatives by reactions with DIB-ET in the presence of Pd and Cu catalysts. The reaction yields for bromobenzene, *p*-bromoanisole and *p*-bromotoluene were 56.2, 50.8 and 50.0%, respectively, calculated by comparing the peak heights of the reaction products and authentic derivatives. These reaction yields were higher than the yields obtained by the reaction between aryl bromides and DPA (20 – 45%) based on the Suzuki coupling reaction.[8] On the other hand, the reaction yields for iodobenzene, *p*-iodoanisole, *p*-iodotoluene, chlorobenzene, *p*-chloroanisole and *p*-chlorotoluene were 101.1, 87.4, 85.5, 0.2, 0.4 and 0.4%, respectively. The reactivity order, aryl iodide > aryl bromide >> aryl chloride, was similar with the results of our previous report, which used DPA and Suzuki coupling.[8] The result can be attributed that the order in leaving group ability is iodine > bromine > chlorine.[22]

Validation of proposed the method

Calibration curves were prepared with a standard mixture of aryl bromides and good linearities (r^2 = 0.995) were obtained between the peak height and the concentration of aryl bromides up to 50 μM (Table 1).

The batch-to-batch repeatability of the proposed method was examined at 0.5, 5 and 50 μM of aryl bromides. The relative

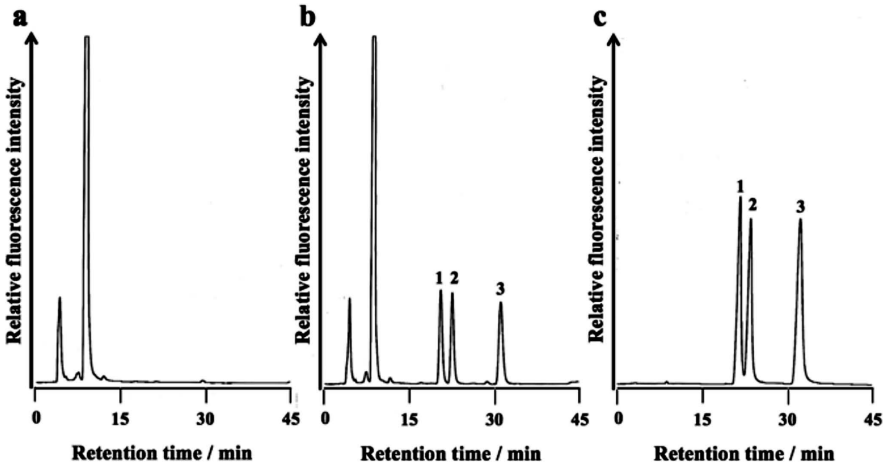

Fig. 4 Chromatograms of (a) reagent blank, (b) reaction mixture and (c) synthesized aryl bromide derivatives. Peaks: 1, *p*-bromoanisole derivative; 2, bromobenzene derivative; 3, *p*-bromotoluene derivative. Sample concentration: 50 μM for all compounds.

Table 1 Calibration curves and detection limits for aryl bromides

Aryl halide	Calibration curve			LOD[b]/nM (fmol/injection)
	Range/μM	Equation[a] (mean ± SD, n = 3)	r^2	
Bromobenzene	0.125 - 50	$Y = (0.4822 \pm 0.0530)X + (0.1495 \pm 0.0664)$	0.995	19 (75)
p-Bromoanisole	0.0625 - 50	$Y = (0.4828 \pm 0.0620)X + (0.2068 \pm 0.1200)$	0.995	14 (56)
p-Bromotoluene	0.125 - 50	$Y = (0.4113 \pm 0.0572)X + (0.2205 \pm 0.0405)$	0.995	23 (92)

a. Y = Peak height (cm); X = concentration (μM). b. S/N = 3.

Table 2 Intra- and inter-day accuracy and precision of the proposed method

Aryl halide	Concentration/ μM	Precision (batch-to-batch RSD, %)		Accuracy, %	
		Within-day (n = 5)	Between-day (n = 5)	Within-day (n = 3)	Between-day (n = 3)
Bromobenzene	0.5	5.2	6.7	88.6	123
	5	2.8	8.9	121	137
	50	6.8	3.3	110	111
p-Bromoanisole	0.5	8.5	9.9	85.3	95.4
	25	3.2	9.3	108	101
	50	6.5	3.9	107	107
p-Bromotoluene	0.5	6.6	8.9	127	131
	5	3.9	7.5	94.5	90.3
	50	7.8	6.2	115	111

standard deviations for intra-day (n = 5) and inter-day (n = 5) runs were less than 8.5 and 9.9%, respectively (Table 2).

The detection limits (S/N = 3) of bromobenzene, *p*-bromoanisole and *p*-bromotoluene of the proposed method were 19, 14, and 23 nM (75, 56 and, 92 fmol/injection) respectively, while those of the previous method with DPA[8] were 350, 50, and 350 nM (1.4, 0.2, and 1.4 pmol/injection) respectively. In this way, the Sonogashira coupling derivatization provided a higher sensitivity for aryl bromides than the Suzuki coupling derivatization despite the fact that both fluorescence derivatives have the same lophine moiety. Likewise, the analytical performances including LODs of the proposed method were compared with those of previously reported LC and GC methods for the determination of aryl halides. The sensitivities of the proposed method towards *p*-bromoanisole and *p*-bromotoluene were nearly equivalent to those of the previous method that used phenyl boronic acid as a fluorogenic derivatization reagent.[23]

Comparing the reactivity of substituted aryl halides with DIB-ET (Sonogashira coupling) vs. DPA (Suzuki coupling)

The influence of different substituent positions on the reactivity of DIB-ET to bromoanisole and bromotoluene was compared with that of DPA. As shown in Fig. 5, the reactivity of DPA to *ortho*-substituted aryl bromides were considerably lower than those to *meta*- and *para*-substituted aryl bromides. The low reactivity could be attributed to the steric hindrance during the transmetalation. On the other hand, a sufficient reactivity of DIB-ET to *ortho*-substituted aryl bromides could be obtained. The reaction yield for *o*-bromoanisole was 41.2%, which is similar to that of *p*-bromoanisole.

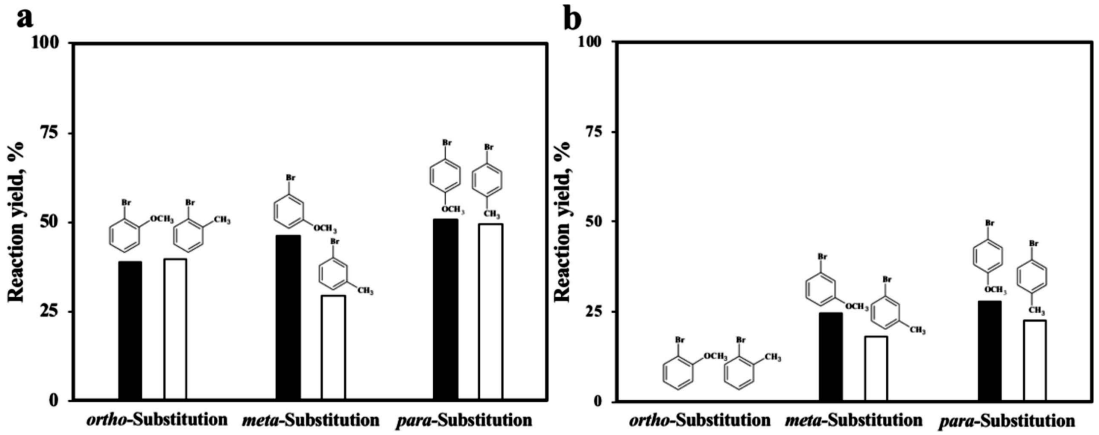

Fig. 5 Reactivities of (a) DIB-ET and (b) DPA to *ortho*-, *meta*-, *para*-substituted bromobenzenes. Sample concentration: 50 μM for all compounds.

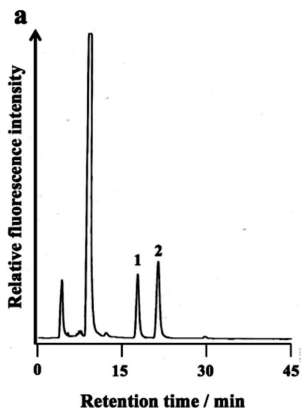

Fig. 6 Chromatograms of bromoanisole derivatives obtained by (a) the reaction with DIB-ET and (b) the reaction with DPA. Peaks: 1, *o*-bromoanisole derivative; 2, *p*-bromoanisole derivative. Sample concentration: 50 μM for all compounds.

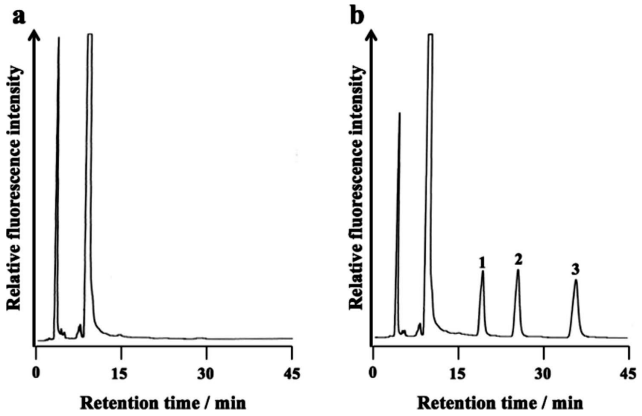

Fig. 7 Chromatograms of (a) blank human serum, (b) human serum spiked with aryl bromides (50 μM serum). Peaks: 1, *o*-bromoanisole derivative; 2, bromobenzene derivative; 3, *o*-bromotoluene derivative.

Figure 6 compares chromatograms of *o*- and *p*-bromoanisole derivatives obtained by the reaction with DIB-ET (Fig. 6a) with those obtained by the reaction with DPA (Fig. 6b). Thus, both *o*- and *p*-bromoanisole derivatives with DIB-ET could be clearly detected on the chromatogram while it was difficult to detect the bromoanisole derivatives with DPA. As we expected, sufficient reactivity for *ortho*-substituted aryl halides was obtained by the proposed fluorescence derivatization technique based on the Sonogashira coupling reaction.

Application of the proposed method for analysis of aryl bromides in human serum

As an application of the proposed method to biological analysis, the proposed method was applied for the determination of aryl bromides in spiked human serum samples. As shown in Fig. 7, the peaks of *o*-bromoanisole, bromobenzene and *o*-bromotoluene derivatives could be clearly detected on the chromatogram with no interference from the serum components. The results indicated that the proposed derivatization reaction could proceed well even in the presence of biological matrices. Also, the recoveries of human serum spiked aryl bromides were varied from 84.6 to 107%, calculated by comparing the peak heights of the reaction products from spiked human serum and the standard solution of aryl bromides (Table 3). Since the proposed method allowed the selective determination of aryl bromides in serum by only simple extraction procedures, it should be useful to monitor toxic aryl bromide levels in biological samples to control and detect occupational and/or accidental exposure to them.

Table 3 Recoveries of human serum spiked with aryl bromides

Aryl halide	Concentration/μM	Recovery, %
Bromobenzene	0.5	107
	5	88.0
	50	85.7
o-Bromoanisole	0.5	102
	5	85.7
	50	96.4
o-Bromotoluene	0.5	90.0
	5	84.6
	50	89.3

Conclusions

In the present study, we reported a novel fluorescence derivatization technique for aryl halides based on the Sonogashira coupling reaction using the fluorescent alkyne, DIB-ET. The proposed derivatization technique allowed for more high sensitive detection of aryl bromides than the previous derivatization technique based on the Suzuki coupling reaction. Moreover, it is noteworthy that the proposed technique could improve the reactivity to *ortho*-substituted aryl halides, which was the limitation of the previous technique. Furthermore, it was confirmed that the proposed method was sufficiently selective toward aryl halides in the presence of other biological components. Therefore, the proposed technique will be a promising tool for the development of a sensitive and selective HPLC analytical method for *ortho*-substituted aryl halides, such as iodotyrosine and thyroid hormones. The development of an analytical method for the determination of thyroid hormones in biological samples based on the Sonogashira coupling is a current interest and an ongoing research in our laboratory.

Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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