Long-wavelength fluorogenic derivatization of aryl halides based on the formation of stilbene by Heck reaction with vinylbenzenes

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Abstract

The long-wavelength fluorogenic derivatization method for aryl halides was developed based on the stilbene formation by the Heck coupling reaction between aryl halides and vinylbenzenes in the presence of palladium (II) acetate as a catalyst. Fluorescent maximum wavelengths of the derivative obtained by the proposed reaction were 365-450 nm, which were 50-100 nm longer than those of the biphenyl derivatives formed with the our previously developed fluorogenic derivatization method. Also, by the investigation using vinylbenzenes that containing electron-donating or -withdrawing functional groups, an internal charge transfer system could contribute to extend emission wavelength of the derivative. Furthermore, the proposed reaction was applied to develop a pre-column derivatization HPLC with fluorescence detection method for aryl bromides using 4-vinylanisole.  

*p*-Substituted aryl bromides (*i.e.*, *p*-bromobenzonitrile, *p*-bromoanisole, bromobenzene, *p*-bromobenzoic acid ethyl ester, *p*-bromotoluene) derivatives were successfully detected within 40 min with the detection limit of 0.007-0.264 µM. Despite the short reaction time of 10 min, the reaction yields for *p*-bromoanisole and bromobenzene were good at 101% and 87%, respectively.

**Keywords:** fluorogenic derivatization, Heck reaction
Introduction

Fluorescence detection method have been applied to trace analysis in combination with high-performance liquid chromatography (HPLC) because of it has high sensitivity and selectivity. However, most compounds are non-fluorescent or weakly fluorescent. Thus, in order to convert from non-fluorescent or weakly compounds to fluorescent ones, fluorescent derivatization techniques have frequently been utilized. Until now, various fluorescence derivatization reagents for typical functional groups such as amine, phenol, and carbonyl groups have been developed. However, fluorescence derivatization can be difficult if the target molecules do not have typical functional groups that can be derivatized. Among such compounds that are difficult to derivatize, our research group have focused on aryl halides. We previously developed a fluorescence derivatization method for aryl halides based on the Suzuki coupling reaction, which is a palladium catalyzed cross-coupling reaction between aryl halide and aryl boronic acid. In this method, we employed fluorescent aryl boronic acid, 4-(4,5-diphenyl-1H-imidazole-2-yl) phenylboronic acid (DPA), as a derivatization reagent. DPA can react selectively with aryl halides to produce fluorescent derivatives even in the presence of numerous biogenic substances. So far, we successfully applied DPA for the monitoring of various aryl halide drugs including; clofibrate, haloperidol and hydroxyzine, in body fluids. However, DPA itself has strong fluorescence, which can interfere with detection of the target derivative. To overcome this issue, we developed a fluorogenic derivatization reaction for aryl halides based on the formation of biphenyl with non-substituted phenylboronic acid (PBA) as a reagent (Fig. 1). The derivatization with PBA did not cause interfering blank peaks derived from excess derivatization reagent. However, the formed fluorescent biphenyl having a fluorescence emission maximum at the short-
wavelength region of 315-350 nm, and is susceptible to interference by coexisting materials.

In the present study, we attempted to develop a long-wavelength fluorogenic derivatization reaction for aryl halides based on the formation of stilbene by Heck coupling reaction (Fig. 1). Heck coupling is a palladium-catalyzed cross-coupling reaction between aryl halide and vinylbenzene to form stilbene derivative.\textsuperscript{14,15} It can be expected that longer conjugated system of stilbene than biphenyl provide long-wavelength fluorescence.\textsuperscript{16,17} Therefore, we evaluated vinylbenzenes as a long-wavelength fluorogenic derivatization reagent for aryl halides. Firstly, the fluorescence spectra of the reaction mixture of aryl bromides with vinylbenzene were measured and compared with those of biphenyls. In addition to non-substituted vinylbenzene, 4-vinylanisole containing electron donating group and 4-(trifluoromethyl)vinylbenzene (4-TFV) containing electron withdrawing group were also tested to extend emission wavelength of the derivative induced by internal charge transfer (ICT).\textsuperscript{17-19} Subsequently, we established a HPLC with fluorescence detection method for aryl bromides after pre-column derivatization with 4-VA.

**Experimental**

**Material and reagents**

4-VA was purchased from Ark Pharm (Illinois, USA). Vinylbenzene, \textit{p}-bromobenzonitrile (Br-Ph-CN), \textit{p}-bromoanisole (Br-Ph-OCH\textsubscript{3}), bromobenzene (Br-Ph-H), \textit{p}-bromobenzoic acid ethyl ester (Br-Ph-COOC\textsubscript{2}H\textsubscript{5}), \textit{p}-bromotoluene (Br-Ph-CH\textsubscript{3}), 4-methoxy-\textit{trans}-stilbene and 4,4'-dimethoxy-\textit{trans}-stilbene were from Tokyo Chemical Industries (Tokyo, Japan). 4-(Trifluoromethyl)vinylbenzene (4-TFV) were from...
Oakwood products (South Carolina, USA). Palladium (II) acetate (Pd(OAc)$_2$), $N,N$-dimethylacetamide (DMAc) were from Nacalai Tesque (Osaka, Japan). Triethylamine (TEA) was purchased from Wako (Tokyo, Japan). Acetonitrile was from Kanto Chemical (Tokyo, Japan).

**Derivatization procedures**

To the DMAc solution including aryl halides (50 µL), 15 mM 4-vinylanisole in DMAc (50 µL), 10 mM Pd(OAc)$_2$ in DMAc and aqueous solution of 60 mM TEA (50 µL) were successively added and mixed in an amber-colored screw-capped vial. After purging with N$_2$ (5.0 mL/s) for 10 sec, the reaction mixture was heated at 100°C for 10 min to form fluorescent derivative.

**HPLC system and conditions**

The HPLC system consisted of a pump LC-20A (Shimadzu, Kyoto, Japan), a Shimadzu RF-20A fluorescence detector, a 7125 injector with a 20-µL loop (Rheodyne, Cotati, CA), and a Chromato-PRO (RTC, Tokyo) as a recorder. Chromatographic separation was performed on a Cosmosil 5C18AR-II (250 × 4.6 mm, i.d., Nakalai Tesque, Kyoto) via isocratic elution with a mixture of acetonitrile and water (60:40, v/v) at a flow rate of 1.0 mL/min. The fluorescence wavelength of the detector was changed according to the time program to detect each derivative at a maximum wavelength (Table S1, Supporting information). After filtration through Hawach Scientific (Shaanxi, China) 0.45-µm PTFE membrane filter, a 20-µL aliquot of the derivatization reaction mixture was injected into the HPLC system.

**Measurement of fluorescence spectra of the derivatives**
The fluorescence spectra of the derivative were investigated after the purification in order to minimize the influence of the fluorescence derived from the reagent blank. The formed derivative in the reaction mixture was purified by HPLC-UV system at 254 nm under the separation conditions described above. The fluorescence excitation and emission spectra of the peak fraction were measured with Shimadzu RF-1500 spectrofluorometer.

**Results and discussion**

*Comparison of fluorescence spectra of the reaction mixture*

Figure 2 shows the fluorescence spectra of the reaction mixture of Br-Ph-COOEt with 4-VA. The fluorescence was observed at excitation and emission maximum wavelength of 320 and 450 nm, respectively. This result presented that Br-Ph-COOEt was derivatized to fluorescent compound by the reaction with 4-VA. While, the intense fluorescence was not observed from the reagent blank. In the reaction solution of the aryl halide and vinylbenzene, there was no significant change in the fluorescence intensity depending on the ratio of water. Table 1 lists the fluorescence excitation and emission maxima of derivative obtained by the reaction of five aryl bromides with vinylbenzene, 4-VA and 4-TFV. On the other hand, the fluorescence excitation/emission maxima (nm) of the Br-Ph-CN, Br-Ph-OCH₃, Br-Ph-COOEt and Br-Ph-CH₃ after the reaction with PBA were reported at 285/335, 280/330, 290/350 and 275/315, respectively. The derivatives formed with vinylbenzene was found to have a fluorescence maximum wavelength on the long wavelength side of 35-45 nm compared with PBA. This longer wavelength emission could be attributed to the difference of conjugated system between stilbene and biphenyl. The derivatives formed with 4-VA and 4-TFV also gave longer wavelength
emission than biphenyl derivatives. It should be noted that the further extension of emission wavelength was observed from the derivatives formed from 4-VA and aryl bromides containing electron withdrawing group (Br-Ph-CN and Br-Ph-COOEt). The similar red shift was also observed from the derivative between 4-TFV and aryl bromides containing electron donating group. These extension of emission wavelength could be brought due to the ICT between electron donating and withdrawing groups in the formed stilbene derivative. Among tested vinylbenzenes, 4-VA could provide the derivative that emit the longest wavelength fluorescence. Consequently, we selected 4-VA as the derivatization reagent for further studies of a development of pre-column derivatization HPLC method.

Chromatographic analysis of the formed aryl halides derivatives of 4-VA

Figure 3 (A) and (B) shows the chromatograms obtained by the injection of the reagent blank (4-VA) and the reaction mixture of aryl bromides with 4-VA into the HPLC system, respectively. Under the HPLC conditions described above, the peaks of the derivatives of Br-Ph-CN, Br-Ph-OCH$_3$, Br-Ph-H, Br-Ph-COOEt and Br-Ph-CH$_3$ were detected at 15, 21, 23, 32 and 36 min, respectively. On the other hand, the peaks considered to be reagent blank were hardly detected because 4-VA should be non-fluorescent. In order to confirm the formation of stilbene derivative by the proposed reaction, the authentic solution of 4,4’-dimethoxy-trans-stilbene and 4-methoxy-trans-stilbene were injected in to the HPLC system. As shown in Fig. 3 (C), the retention times of 4,4’-dimethoxy-trans-stilbene and 4-methoxy-trans-stilbene were identical with those of the derivative of Br-Ph-OCH$_3$ and Br-Ph-H, respectively, which revealed that aryl bromides were converted to stilbenes by the reaction with 4-VA.
Optimization of the reaction condition between 4-VA and aryl halides

In order to get higher reactivity, the derivatization reaction conditions were optimized using a standard solution of aryl bromides. Different kinds of solvents including; DMAc, 1,4-dioxane (Dioxane), acetonitrile (CH$_3$CN), ethanol and N, N-dimethylformamide (DMF) were examined. Among the tested solvents, DMAc yielded the largest peak area of aryl bromide derivatives (Fig. S1). The concentration of 4-VA was investigated over a range of 1-20 mM, and the largest peak area of derivatives were obtained at a concentration of 15 mM (Fig. S2). The concentration of Pd(OAc)$_2$ was investigated over a range of 0.1-12 mM. The maximum and constant peak area of aryl bromides were obtained at more than 8 mM. 10 mM of Pd(OAc)$_2$ was selected (Fig. 4(A)). Different kinds of bases including; TEA, N,N-diisopropylethylamine (DIPEA), potassium fluoride (KF), and sodium hydrogen phosphate (Na$_2$HPO$_4$) were tested. As shown in Fig. S3, the addition of base could improve the peak area of the derivatives except for Na$_2$HPO$_4$. Among the tested bases, TEA provided the largest peak area of aryl bromide derivatives. Then, the concentration of TEA was investigated over a range of 0-70 mM, and the largest or constant peak area of aryl bromides were obtained at 60 mM. Therefore, 60 mM of TEA was selected (Fig. 4(B)). The effects of the reaction temperature and time were investigated, and optimum conditions were achieved with heating at 100 °C for 10 min (Fig. S4 and S5).

Under the optimized conditions, the reaction yields of Br-Ph-OCH$_3$ and Br-Ph-H, were calculated by comparing the peak areas of the reaction products with those of authentic 4,4’-dimethoxy-trans-stilbene and 4-methoxy-trans-stilbene. The estimated reaction yields of Br-Ph-OCH$_3$ and Br-Ph-H were 101 and 87%, respectively, and therefore it was confirmed that the proposed derivatization reaction proceeded with excellent yield.
Validation of proposed the method

 Calibration curves were prepared with a standard mixture of aryl bromides and good linearities ($r^2 > 0.997$) were obtained between the peak height and the concentration of aryl bromides in the range of 1.0-250 µM except for p-bromoanisole (0.05-25 µM) (Table 2). The detection limits of aryl bromides calculated at a signal-to-noise ratio (S/N) of 3 were ranged from 0.007 to 0.264 µM. The optimum reaction conditions and the sensitivities of the proposed method were compared to those of the previously developed methods by our research group (Table 4). The sensitivities of the proposed method were relatively superior than those of the DPA. Although the proposed method was less sensitive than the PBA method except for Br-Ph-OCH$_3$, the longer wavelength fluorescence emission should be effective to reduce the interfering peaks derived from co-existing components. In addition, the proposed method could provide the excellent reaction yields within shorter reaction time. The repeatability of the proposed method was examined using three different concentrations of aryl bromides in the calibration range (25, 150 and 250 µM). The relative standard deviations for within-day ($n = 5$) and between-day ($n = 5$) runs were less than 7.7 and 8.6%, respectively (Table 3), and therefore sufficient repeatability of the proposed method could be confirmed.

Conclusion

 In this study, we reported a long-wavelength fluorogenic derivatization reaction for aryl halides based on the formation of stilbene by Heck coupling reaction. Since the maximum fluorescence wavelengths of the derivatives obtained by the proposed method using 4-VA were 50-100 nm longer than those of the derivative obtained by the previous
method using PBA, it should be expected to reduce the interfering peaks derived from
autofluorescence of samples. Moreover, it should be noteworthy that the proposed
derivatization reaction could proceeds with highly reaction yields (>87%) within a short
time (10 min). Hence, by taking advantage of the long wavelength and short reaction
time, the proposed method will be a promising tool for the development of post-column
derivatization HPLC system for aryl halides.

Supporting information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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Table 1  Fluorescence properties of derivatives obtained by the reaction of aryl bromides with 4-VA, vinylbenzene and 4-TFV

| Aryl bromide | 4-VA | Vinylbenzene | 4-TFV |
|--------------|------|--------------|-------|
|              | λex  | λem          | λex  | λem | λex  | λem |
|              | (nm) | (nm)         | (nm) | (nm) | (nm) | (nm) |
| Br-Ph-OCH₃   | 290  | 370          | 290  | 370 | 305  | 400 |
| Br-Ph-CH₃    | 290  | 365          | 290  | 350 | 300  | 370 |
| Br-Ph-H      | 290  | 370          | 290  | 340 | 290  | 350 |
| Br-Ph-COOEt  | 320  | 450          | 310  | 390 | 300  | 360 |
| Br-Ph-CN     | 320  | 435          | 300  | 380 | 300  | 355 |
Table 2  Calibration curves and detection limits for aryl bromides

| Aryl halide     | Calibration curve | LOD$^b$ (µM (pmol/injection)) |
|-----------------|-------------------|-------------------------------|
|                 | Range (µM)        | Equation$^a$                   | $r^2$ |
|                 |                   | (mean±SD, n = 3)               |      |
| Br-Ph-CN        | 1-250             | $Y = (3.14 \times 10^2 \pm 36.2) X + (6.38 \times 10^2 \pm 1.29 \times 10^2)$ | 0.999 | 0.264 (1.3) |
| Br-Ph-OCH$_3$   | 0.05-25           | $Y = (7.10 \times 10^3 \pm 8.83 \times 10^2) X + (5.90 \times 10^3 \pm 2.29 \times 10^2)$ | 0.998 | 0.007 (0.04) |
| Br-Ph-H         | 1-250             | $Y = (3.81 \times 10^2 \pm 40.3) X + (2.05 \times 10^2 \pm 72.0)$ | 0.999 | 0.120 (0.60) |
| Br-Ph-COOEt     | 1-250             | $Y = (4.69 \times 10^3 \pm 63.0) X + (1.14 \times 10^3 \pm 7.62 \times 10^2)$ | 0.997 | 0.094 (0.47) |
| Br-Ph-CH$_3$    | 1-250             | $Y = (9.01 \times 10^3 \pm 1.18 \times 10^2) X + (2.13 \times 10^3 \pm 6.14 \times 10^2)$ | 0.998 | 0.073 (0.37) |

$^a$ $Y = $ Peak height; $X = $ Concentration (µM)

$^b$ S/N=3
Table 3  Intra- and inter-day precision of the proposed method

| Aryl bromide | Concentration, µM | Within-day (n = 5) | Between-day (n =5) |
|--------------|-------------------|-------------------|-------------------|
| Br-Ph-CN     | 25                | 1.2               | 1.0               |
|              | 150               | 1.0               | 2.3               |
|              | 250               | 2.4               | 6.1               |
| Br-Ph-OCH₃   | 2.5               | 4.9               | 6.7               |
|              | 15                | 5.7               | 5.1               |
|              | 25                | 7.7               | 2.9               |
| Br-Ph-H      | 25                | 5.2               | 8.6               |
|              | 150               | 2.4               | 7.4               |
|              | 250               | 3.7               | 4.6               |
| Br-Ph-COOEt  | 25                | 0.8               | 2.7               |
|              | 150               | 1.9               | 5.4               |
|              | 250               | 1.8               | 6.7               |
| Br-Ph-CH₃    | 25                | 7.3               | 6.1               |
|              | 150               | 5.7               | 5.5               |
|              | 250               | 4.8               | 4.0               |
Table 4  Comparison of the derivatization conditions and LODs obtained with various fluorescence derivatization reagent for aryl bromides

| Reagent | 4-VA<sup>a</sup> | DPA<sup>b</sup> | PBA<sup>c</sup> |
|---------|-----------------|----------------|----------------|
| Derivatization condition | 100℃, 10 min | 100℃, 45 min | 100℃, 30 min |
| LOD, µM |                 |                |                |
| Br-Ph-CN | 0.264 | - | 0.003 |
| Br-Ph-OCH<sub>3</sub> | 0.007 | 0.050 | 0.013 |
| Br-Ph-H | 0.120 | 0.35 | - |
| Br-Ph-COOEt | 0.094 | - | 0.007 |
| Br-Ph-CH<sub>3</sub> | 0.073 | 0.35 | 0.030 |

<sup>a</sup> The proposed method.

<sup>b</sup> N. Kuroda, S. Sugihara, Y. Sugihara et al., Journal of Chromatography A 1066 (2005) 119-125.

<sup>c</sup> N. Kishikawa, K. Kubo, S. F. Hammad et al., Journal of Chromatography A 1216 (2009) 6873-6876.
Figure captions

Fig. 1  Scheme of fluorogenic derivatization for aryl halide by (a) the reaction with phenylboronic acid (PBA) based on the Suzuki coupling reaction and (b) the reaction with vinylbenzenes based on the Heck coupling reaction.

Fig. 2  Fluorescence spectra obtained from the reaction mixture of Br-Ph-COOEt with 4-VA and the reagent blank. Excitation spectra with the emission wavelength at 450 nm of the product (A, solid line) and the reagent blank (B, dashed line). Emission spectra with the excitation wavelength at 320 nm of the product (C, solid line) and the reagent blank (D, dashed line). Solvent: acetonitrile / water = 60 / 40 (v/v%)

Fig. 3  Chromatograms of (A) reagent blank, (B) reaction mixture and (C) authentic stilbenes. Peaks: 1, Br-Ph-CN derivative; 2, Br-Ph-OCH₃ derivative; 3, Br-Ph-H derivative; 4, Br-Ph-COOEt; 5, Br-Ph-CH₃; 2’, 4,4’-dimethoxy-trans-stilbene; 3’, 4-methoxy-trans-stilbene. Sample concentration: 100 µM except for Br-Ph-OCH₃ (Br-Ph-OCH₃ 10 µM).

Fig. 4  Effect of the (a) Pd(OAc)₂ and (b) TEA concentrations on the peak area of aryl bromides. Compounds: ■, p-bromoanisole; ◊, p-bromotoluene; ▲, p-bromobenzoic acid ethyl ester; □, bromobenzene; ●, p-bromobenzonitrile. Sample concentration: 30 µM for all compounds.
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Fig. 4  Effect of the (a) Pd(OAc)$_2$ and (b) TEA concentrations on the peak area of aryl bromides. Compounds: ■, $p$-bromoanisole; ◇, $p$-bromotoluene; ▲, $p$-bromobenzoic acid ethyl ester; □, bromobenzene; ●, $p$-bromobenzonitrile. Sample concentration: 30 µM for all compounds.
Graphical index

**PBA (Suzuki coupling, previous method)**

\[
\text{R-Br} + \text{HO-B} \xrightarrow{\text{Pd(OAc)}_2, \text{Base}} \text{R-} \quad \text{Biphenyl derivative (fluorescence)}
\]

R = CN, OCH₃, COOEt, CH₃

PBA (non-fluorescence)

**Short wavelength**

λₑᵐ = 315-350 nm

**4-VA (Heck coupling, proposed method)**

\[
\text{R-Br} + \text{OCH₃} \xrightarrow{\text{Pd(OAc)}_2, \text{Base}} \text{R-} \quad \text{Stilbene derivative (fluorescence)}
\]

R = CN, OCH₃, H, COOEt, CH₃

4-VA (non-fluorescence)

**Long wavelength**

λₑᵐ = 365-450 nm