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Journal of Chromatography A, 982 (2002) 111–118
JOURNAL OF CHROMATOGRAPHY A
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Development of hydrophilic fluorogenic derivatization reagents for thiols: 4-(*N*-acetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole and 4-(*N*-trichloroacetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

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Received 4 June 2002; received in revised form 14 August 2002; accepted 14 August 2002

Abstract

Sensitive, reactive, and hydrophilic fluorogenic reagents for thiols with the benzofurazan skeleton, 4-(*N*-acetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (AcABD-F) and 4-(*N*-trichloroacetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (TCAcABD-F) have been developed. These reagents reacted with thiols within 10 min at 60 °C. AcABD-F and TCAcABD-F themselves do not fluoresce but are strongly fluorescent after the reaction with thiol compounds. The generated derivatives were highly water-soluble, since they dissociated a proton and ionized in the neutral pH region. The derivatives with four biologically important thiol compounds were separated on a reversed-phase HPLC column and detected fluorometrically at 504 nm with excitation at 388 nm. The detection limit attained for homocysteine with AcABD-F was 25 fmol on column (11 n*M*) (signal-to-noise ratio=3), and that for glutathione with TCAcABD-F was 45 fmol on column (20 n*M*).

Keywords: Derivatization, LC; Thiols

1. Introduction

Thiol compounds such as glutathione (GSH), homocysteine (Hcy) and, *N*-acetylcysteine (Nac) have biologically important activities. For instance, an elevated plasma homocysteine level is supposed to be a risk factor for vascular diseases [1–3]. Glutathione plays an important role as an antioxidant which protects cells against reactive oxygen species (ROS) [4,5]. Thiol compounds, however, exist in small quantities in biological samples, and thus a sensitive and selective determination method for these compounds is required in the field of clinical sciences.

High-performance liquid chromatography (HPLC) combined with fluorometric detection is the most popular method because it is sensitive and selective. Biological thiol compounds such as cysteine, homocysteine, glutathione, and *N*-acetylcysteine are non-fluorescent; thus many derivatization reagents for

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01274-8

these compounds have been developed such as the following: (I) *N*-substituted maleimide reagents [6–11], (II) bimane reagents [12], and (III) dansyl-aziridine [13,14]. These reagents, however, are not totally satisfactory, since (I) *N*-substituted maleimide reagents generate multiple fluorescent products, which interfere with quantification of thiols, (II) bimane reagents are not selective for thiols as they also react with other functional groups such as amino groups, and (III) dansyl-aziridine has its own fluorescence that interferes with the detection of generated derivatives in HPLC analysis. Furthermore, the reactivity of dansyl-aziridine is very low and it takes as long as 60 min to derivatize thiol compounds (pH 8.2, 60 °C) [13,14].

So far we have developed fluorescent derivatization reagents for thiols having the benzofurazan skeleton [15] such as ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) [16,17], 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [17,18], and 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [19]. The benzofurazan (2,1,3-benzoxadiazole) reagents have the following advantages: (I) the reagents themselves are nonfluorescent and the generated products are strongly fluorescent, (II) the reactivity of these reagents are rather high because its molecular mass is small and, (III) the derivatives have long excitation and emission wavelengths [15]. SBD-F, the most popular derivatization reagent for thiols, is quite sensitive because of its high quantum yield. The solubility of the derivatives in water is high since SBD-F has a sulfonate group and it ionizes in water. SBD-F is, however, not suitable for rapid analyses because the derivatization reaction takes 60 min (pH 9.5, 60 °C) [16]. Although ABD-F and DBD-F are more reactive and the derivatization reaction takes less than 10 min, they are less sensitive than SBD-F [16,18]. In addition, the solubility of the derivatives of high-molecular-mass substances with DBD-F and ABD-F seems to be low in water because they do not ionize. Thus, the development of sensitive, selective, and reactive derivatization reagents is still desired.

The purpose of our study was to develop new benzofurazan derivatization reagents that have the following characteristics: (I) the reactivity to thiols is higher than ABD-F, (II) the fluorescence intensity of the derivatives is stronger than SBD-F, and (III) the derivatives ionize and are highly soluble in water of neutral pH like SBD-F.

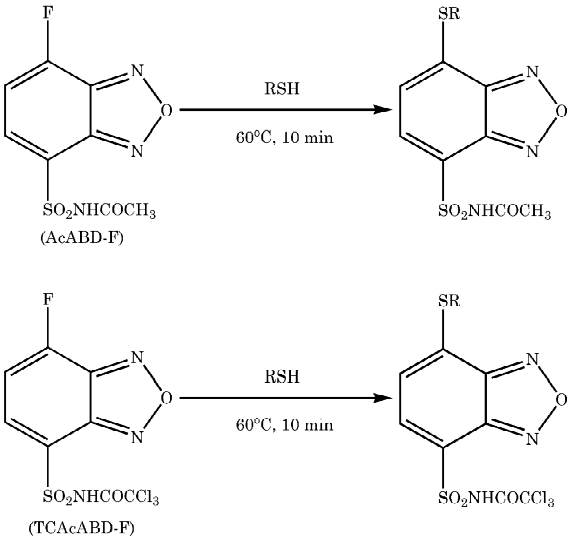

Fig. 1. Chemical structures of AcABD-F, TCAcABD-F, and their reaction with thiols.

In this paper we report the development of two new fluorogenic derivatization reagents for thiols, 4-(*N*-acetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (AcABD-F) and 4-(*N*-trichloroacetylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (TCAcABD-F) (Fig. 1). The reactivity of the reagents as well as the detectability of thiol compounds was investigated.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, dichloromethane (CH_2Cl_2), ethyl acetate (AcOEt), *n*-hexane, and methanol (CH_3OH) (HPLC grade) were purchased from Kanto (Tokyo, Japan). Thiols (glutathione, cysteine, homocysteine, and *N*-acetylcysteine) were obtained from Wako (Osaka, Japan), Kyowahakkoh (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan) and Wako, respectively. ABD-F was purchased from Wako. Tetraethylammonium bromide was obtained from Aldrich (Milwaukee, WI, USA). Water was purified on a Mili-Q reagent system (Millipore, Bedford, MA,

USA). All other reagents were of guaranteed reagent grade and used without further purification.

2.2. Apparatus

Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured by a JEOL LA-500 spectrometer (Tokyo, Japan) with tetramethylsilane as the internal standard in $CDCl_3$. Mass spectra were measured using a Hitachi M-1200H mass spectrometer (atmospheric pressure chemical ionization (APCI) system) (Tokyo, Japan). UV–vis absorption spectra were measured using a Jasco Ubest-50 spectrometer (Tokyo, Japan). Fluorescence spectra were measured by a Hitachi F-4500 fluorescent spectrometer.

2.3. HPLC system

The HPLC system consisted of a Hitachi L-7100 pump, Rheodyne injection valve with a 200-μl filling loop, a Hitachi L-7480 fluorescent detector, and a Hitachi D-7500 integrator. The separation of the derivatives was studied using an analytical column, TSKgel ODS-80 Ts (150×4.6 mm; I.D., 5 μm) (Tosoh, Tokyo, Japan) and isocratic elution [acetonitrile–67 m*M* phosphate buffer, pH 7, (4:96, v/v)] containing 5 m*M* tetraethylammonium bromide for the detection of AcABD-thiols, [acetonitrile–67 m*M* phosphate buffer, pH 7 (20:80, v/v)] containing 5 m*M* tetraethylammonium bromide for the detection of TCAcABD-thiols, at a flow-rate of 1.0 ml/min. The eluate was monitored by fluorescence detection (λ_{ex} 388 nm; λ_{em} 504 nm).

2.4. Syntheses

2.4.1. Synthesis of AcABD-F

ABD-F (103 mg) was dissolved in 5 ml of acetic anhydride. The mixture was stirred at 150 °C for 150 min and evaporated under reduced pressure. The residue was chromatographed on silica gel [eluent, CH_2Cl_2–CH_3OH (95:5, v/v)] to afford AcABD-F (84.6 mg, 69%) as a pale yellow powder: δ_H 8.16 (d, 1H, J=7.4), 7.28 (d, 1H, J=7.4), 1.85 (3H, s). APCI-MS: m/z 258 [$(M-H)^-$].

2.4.2. Synthesis of 4-(N-acetylaminosulfonyl)-7-methylthio-2,1,3-benzoxadiazole (AcABD-SMe)

AcABD-F (29.9 mg) in 2 ml of acetonitrile was added to saturated $NaHCO_3$ solution (1.5 ml). To the mixture 2 ml of 15% $NaSCH_3$ solution in 3 ml of acetonitrile was slowly dropped and stirred at room temperature for 50 min. Then the alkaline solution was neutralized with 7 ml of 2 *M* HCl and extracted by CH_2Cl_2 (50 ml, 3 times). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on silica gel [eluent, CH_2Cl_2–CH_3OH (95:5, v/v)] to afford AcABD-SMe (24.6 mg, 74%) as a pale yellow powder: δ_H 8.01 (1H, d, J=7.7), 7.17 (1H, d, J=7.7), 2.01 (3H, s), 1.84 (3H, s). APCI-MS: m/z 286 [$(M-H)^-$], 288 [$(M+H)^+$].

2.4.3. Synthesis of 4-(N-methylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

Forty percent solution of methylamine in water (15 ml) was added to 4-chlorosulfonyl-7-fluoro-2,1,3-benzoxadiazole (CBD-F) [18] (306 mg) in 50 ml of acetonitrile. Then the alkaline solution was neutralized with 2 *M* HCl and extracted using CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on silica gel [(eluent, CH_2Cl_2, CH_2Cl_2–CH_3OH, (95:5, v/v)] to afford 4-(*N*-methylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (100 mg, 33.2%) as a pale yellow powder: δ_H 8.04–8.09 (1H, m), 7.14–7.26 (1H, m), 4.98 (1H, s), 2.67–2.76 (3H, m). APCI-MS: m/z 231 [$(M-H)^-$]. Anal. Calcd. for $C_7H_6N_3O_3S$: C, 36.36; H, 2.62; N, 18.17. Found: C, 36.23; H, 2.71; N, 17.92.

2.4.4. Synthesis of 4-(N-methylaminosulfonyl)-7-methylthio-2,1,3-benzoxadiazole

To 34.6 mg of 4-(*N*-methylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole in 5 ml of acetonitrile was added dropwise 3 ml of 15% $NaSCH_3$ solution containing 1.5 ml of saturated $NaHCO_3$ solution. The mixture was stirred at room temperature for 60 min. Then the alkaline solution was neutralized with 6 ml of 1 *M* HCl and extracted using CH_2Cl_2 (50 ml, 3 times). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on silica gel (eluent, CH_2Cl_2) to afford 4-(*N*-methylaminosul-

fonyl)-7-methylthio-2,1,3-benzoxadiazole (38 mg, 97.9%) as a yellow powder: δ_H 7.93 (1H, d, $J=7.4$), 7.02–7.04 (1H, d, $J=7.4$), 4.95 (1H, br), 2.69 (3H, s), 2.68–2.69 (3H, d, $J=5.5$). APCI-MS: m/z 260 [$(M+H)^-$]. Anal. Calcd. for $C_8H_9N_3O_3S_2$: C, 37.05; H, 3.50; N, 16.20. Found: C, 37.07; H, 3.58; N, 15.96.

2.4.5. Synthesis of 4-(N-acetyl-N-methylamino-sulfonyl)-7-methylthio-2,1,3-benzoxadiazole (MeAcABD-SMe)

Pyridine (0.15 ml) was added to 4-(*N*-methylaminosulfonyl)-7-methylthio-2,1,3-benzoxadiazole (38 mg) in 2 ml of acetic anhydride. After the addition the mixture was stirred at 85 °C for 150 min. Then the alkaline solution was acidified with 4 ml of 1 *M* HCl and extracted using AcOEt (50 ml, twice), followed by addition of saturated $NaHCO_3$ (50 ml) and extracting with AcOEt (50 ml, twice). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on silica gel [eluent, CH_2Cl_2–*n*-hexane (3:1, v/v)] to afford MeAcABD-SMe (33 mg, 86.8%) as a yellow powder: δ_H 8.08 (1H, d, $J=7.3$), 7.05 (1H, d, $J=7.3$), 3.40 (3H, s), 2.70 (3H, s), 2.40 (3H, s). APCI-MS: m/z 301 [$(M+H)^-$].

2.4.6. Synthesis of (TCAcABD-F)

Trichloroacetic anhydride (2 ml) was added to 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (51.3 mg) dissolved in 1 ml of acetonitrile. The mixture was stirred at 130 °C for 150 min. After cooling the reaction mixture, CH_2Cl_2 (100 ml) was added and washed with HCl solution (pH 1.2). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed twice on silica gel [eluent, CH_2Cl_2, AcOEt–CH_3OH (4:1, v/v)] to afford TCAcABD-F (73 mg, 91%) as a pale yellow powder: δ_H 8.20 (dd, 1H, $J=7.6$, J= 4.0), 7.11–7.14 (m, 1H).

2.4.7. Synthesis of 4-(N-trichloroacetylamino-sulfonyl)-7-methylthio-2,1,3-benzoxadiazole (TCAcABD-SMe)

4-Aminosulfonyl-7-methylthio-2,1,3-benzoxadiazole (ABD-SMe) (11 mg) was dissolved in trichloroacetic anhydride (2 ml). The mixture was stirred at 120 °C for 150 min. After CH_2Cl_2 was added to the mixture and washed twice with HCl solution (pH 2), the solution was evaporated under reduced pressure. The residue was chromatographed twice on silica gel [eluent, CH_2Cl_2, CH_2Cl_2–AcOEt–CH_3OH (3:1:0–4:2:1, v/v)] to afford TCAcABD-SMe (17.5 mg, >99%) as pale yellow powder: δ_H 7.87–7.92 (d, 1H, $J=7.4$), 7.16–7.22 (d, 1H, $J=7.4$), 2.61 (s, 3H).

2.5. Spectrometric characteristics of AcABD-SMe, TCAcABD-SMe, and MeAcABD-SMe at various pH values

Fluorescence intensities (FIs) of 30 μ*M* AcABD-SMe, TCAcABD-SMe, and MeAcABD-SMe in various pH solutions (Britton–Robinson buffer for pH 2–12, 0.5 *M* H_2SO_4 for pH 0, 0.05 *M* H_2SO_4 for pH 1) were measured at maximum excitation and emission wavelength for each pH tested.

The UV–vis spectra of AcABD-SMe were measured in various pH solutions (Britton–Robinson buffer for pH 2–12, Walpole buffer for pH 0, 0.25, 0.5, 1, and 1.5). The pK_a value was obtained according to Henderson–Hasselbach Eq. (1) [20].

$$\log [(A_{max} - A)/(A - A_{min})] = pH - pK_a \quad (1)$$

Solution A (400 ml) for the Britton–Robinson buffer consisted of 0.94 ml of 85% phosphoric acid, 0.96 ml of acetic acid, and 0.99 g of boric acid. Solution B was 0.2 *M* NaOH (2.4 g/300 ml). Buffers of various pH values were prepared by mixing appropriate volumes of solutions A and B. The Walpole buffer was prepared by mixing the solution of 1 *M* sodium acetate, a solution of 2 *M* hydrochloric acid, and water.

2.6. Fluorescence intensity of AcABD-SMe, TCAcABD-SMe, ABD-SMe, DBD-SMe and SBD-SMe

The fluorescence spectra of AcABD-SMe, TCAcABD-SMe, ABD-SMe, 4-(*N*,*N*-dimethylaminosulfonyl)-7-methylthio-2,1,3-benzoxadiazole (DBD-SMe), and ammonium 7-methylthio-2,1,3-benzoxadiazole-4-sulfonate (SBD-SMe) (2 μ*M*) in phosphate buffer (pH 7) were obtained.

2.7. Derivatization of thiols

To the 1.5-ml vial was added 100 μl of AcABD-F (5 m*M*) or TCAcABD-F (5 m*M*) in 0.1 *M* borate buffer containing 5 m*M* Na_2EDTA (pH 9.3) and 100 μl of mixed thiols (100 μ*M* each of gluthatione, cysteine, homocysteine, and *N*-acetylcysteine) in 0.1 *M* borate buffer containing 5 m*M* Na_2EDTA (pH 9.3). The vial was capped and heated at 60 °C for 10 min. The vial was cooled in ice–water and acidified with 25 μl of 2 *M* HCl to stop the reaction.

2.8. Calibration curves for the derivatives of AcABD-F and TCAcABD-F with thiols

The derivatization reactions were performed at the condition described above (see Section 2.7). The concentrations of thiols were 45 μ*M*, 4.5 μ*M*, 450 n*M*, 45 n*M*, 22.5 n*M*, 4.5 n*M* each of glutathione, cysteine, homocysteine, and *N*-acetylcysteine.

3. Results and discussion

3.1. Design and syntheses of AcABD-F and TCAcABD-F

In order to develop a reactive reagent, it is necessary to introduce an electron withdrawing group at the *para* position of fluorine substituent, the reactive moiety of the reagent having the benzofurazan skeleton. We selected the acetylaminosulfonyl group and trichloroacetylaminosulfonyl group as such a group, since these groups are more electron withdrawing than the aminosulfonyl group, the substituent group of ABD-F. Thus AcABD-F and TCAcABD-F (Fig. 1) were expected to be more reactive than ABD-F. Furthermore, AcABD-F and TCAcABD-F can ionize in solutions of lower pH than ABD-F because the amide proton of the acetylaminosulfonyl group has a lower pK_a value than that of the amine in the aminosulfonyl group, suggesting that derivatives of AcABD-F and TCAcABD-F would be highly water-soluble in neutral pH solution. AcABD-F and TCAcABD-F were synthesized according to the scheme in Fig. 2 in good yield.

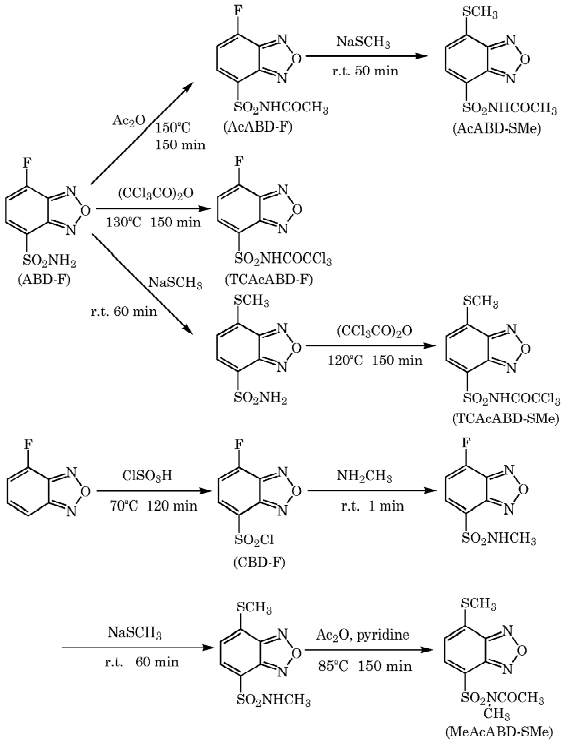

Fig. 2. Synthetic scheme for AcABD-SMe, TCAcABD-SMe, and MeAcABD-SMe.

3.2. Fluorescence characteristics of the derivatives of AcABD-F, TCAcABD-F, and MeAcABD-F

To examine the fluorescence characteristics of the derivatives, the representative derivatives with thiols, AcABD-SMe and TCAcABD-SMe were synthesized according to the scheme in Fig. 2. The absorption and fluorescence spectra of AcABD-F, TCAcABD-F, AcABD-SMe, and TCAcABD-SMe were measured (Table 1). As expected, AcABD-F and TCAcABD-F, the reagents themselves, did not fluoresce; on the contrary AcABD-SMe and TCAcABD-SMe, the generated derivatives, strongly fluoresced.

The excitation and emission wavelengths of AcABD-SMe (λ_{ex} 390 nm; λ_{em} 533 nm) and TCAcABD-SMe (λ_{ex} 391 nm; λ_{em} 530 nm) were longer than the derivatives of the conventional reagents [7–9,11,21]: *N*-(1-pyrene)maleimide (NPM) (λ_{ex} 342 nm; λ_{em} 396 nm), *N*-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM), *N*-(9-acridinyl)-maleimide (NAM) (λ_{ex} 360 nm; λ_{em} 455 nm),

Table 1
Relative fluorescence intensities and maximum excitation and emission wavelengths of benzofurazan reagents

Compounds	λ_{ex} (nm)	λ_{em} (nm)	RFI[a]
AcABD-F	–	–	ND[b]
AcABD-SMe	390	533	117
TCAcABD-F	–	–	ND[b]
TCAcABD-SMe	391	530	197
SBD-SMe	380	530	100[c]
ABD-SMe	389	525	33
DBD-SMe	390	520	23

[a] RFIs were measured at maximum excitation and emission wavelengths for each compounds tested in the phosphate buffer (pH 7).
[b] ND, not detected.
[c] The FI of SBD-SMe was arbitrarily taken as 100.

9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1 - yl)phenyl - 3 - oxo - 3H - naphtho[2, 1 - b]pyran (ThioGlo 3) (λ_{ex} 365 nm; λ_{em} 455 nm), methyl 4-(6-methoxy-2-naphthyl)-4-oxo-2-butenoate (λ_{ex} 310 nm; λ_{em} 450 nm) and almost equivalent to those of monobromobimane (mBBr) (λ_{ex} 338 nm; λ_{em} 540 nm) [12], (1-dimethylaminonaphthalene-5-sulfonyl)aziridine (dansyl-aziridine) (λ_{ex} 388 nm; λ_{em} 540 nm) [13,14] and other benzofurazan reagents such as SBD-F (λ_{ex} 380 nm; λ_{em} 530 nm) [16], ABD-F (λ_{ex} 389 nm; λ_{em} 525 nm) [18], or DBD-F (λ_{ex} 390 nm; λ_{em} 520 nm) [19].

The relationship between the pH of the solution and FIs of the derivatives, AcABD-SMe and TCAcABD-SMe, is shown in Fig. 3. The FIs of AcABD-SMe and TCAcABD-SMe were dependent on the pH of the solution. At pH 6 and above the FI of AcABD-SMe was 12 times stronger than that at pH 2 and below. At pH 2 and above the FI of TCAcABD-SMe was 12 times stronger than that at pH 0 (Fig. 3). It was assumed that the increase in the FI was due to the ionization of AcABD-SMe and/or TCAcABD-SMe. In order to clear this, MeAcABD-SMe, which does not have a proton at the nitrogen atom of the amino group and cannot ionize, was synthesized (Fig. 2) and the effect of pH on the FI examined (Fig. 3). The FI of MeAcABD-SMe was completely independent of pH in contrast to that of AcABD-SMe (Fig. 3). The absorbance of AcABD-SMe as well as its FIs varied in the acidic pH ranges and the pK_a value of AcABD-SMe obtained was 3.6 (Fig. 3). These results indicated that AcABD-SMe completely ionized at pH 6 and above. Because of a small change in the absorbance of TCAcABD-SMe, we could not obtain its pK_a value. However, according to the FI changes versus pH, we speculated that TCAcABD-SMe would ionize at pH 3 and above.

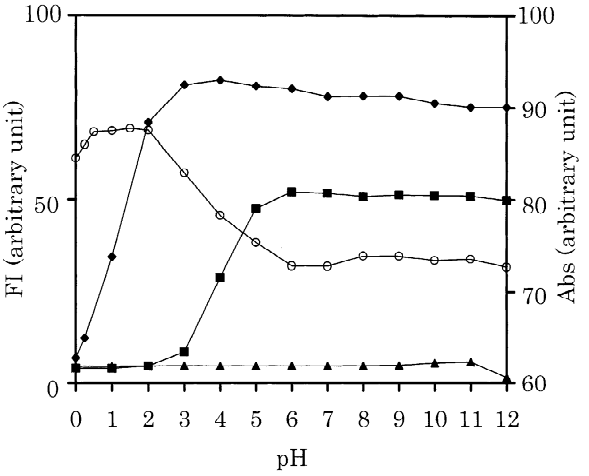

Fig. 3. Effect of pH on the spectrometric characteristics of AcABD-SMe, TCAcABD-SMe, and MeAcABD-SMe. Symbols are the FI of AcABD-SMe(■), the FI of TCAcABD-SMe(♦), the FI of MeAcABD-SMe(▲), and the Abs of AcABD-SMe (○).

Next, we compared the retention times (t_R) of AcABD-SMe and ABD-SMe on the HPLC system. The t_R values were 42.3 and 19 min, respectively, at a mobile phase pH of 1.4, and 13.3 and 22.8 min, respectively, at a mobile phase pH of 7. The t_R of AcABD-SMe became shorter with a mobile phase of pH 7 in stead of pH 1.4, whereas the t_R of ABD-SMe was not changed. This is because the ionized AcABD-SMe is more hydrophilic at pH 7 than nonionized AcABD-SMe in pH 1.4. Generally speaking, ionization changes the electronic state and fluorescence characteristics of molecules. The FIs of AcABD-SMe and TCAcABD-SMe were also increased in their ionized states and the detection of ionized states seemed to be favorable for sensitive detection of AcABD-thiol and/or TCAcABD-thiol derivatives.

3.3. Relative fluorescence intensity (RFI) of AcABD-SMe, TCAcABD-SMe, SBD-SMe, ABD-SMe, and DBD-SMe

The FIs of AcABD- and TCAcABD-thiol derivatives were compared with the derivatives of other

benzofurazan reagents (Table 1). Measurement of FI was carried out at pH 7, at which AcABD-SMe and TCAcABD-SMe produce stronger FIs as they are ionized. As shown in Table 1, the FI of AcABD-SMe was as strong as that of SBD-SMe and was much stronger than those of ABD-SMe and DBD-SMe. The FI of TCAcABD-SMe was even stronger than those of SBD-SMe, ABD-SMe, and DBD-SMe. These results indicated that AcABD-F and TCAcABD-F would be as sensitive as SBD-F and much more sensitive as compared to ABD-F and DBD-F.

3.4. Derivatization of thiols with AcABD-F and TCAcABD-F

Time course studies on the derivatization reaction of thiols (GSH, Cys, Hcy, and Nac) (100 μ*M*) with AcABD-F (5 m*M*) or TCAcABD-F (5 m*M*) were performed at 60 °C (pH 9.3). As shown in Fig. 4A and 4B the peak height reached a plateau after 10 min suggesting that the derivatization reaction proceeded to completion within 10 min. SBD-F and ABD-F require 60 min and 10 min, respectively, for the derivatization reaction; therefore, AcABD-F and TCAcBD-F are much more reactive than SBD-F and as reactive as ABD-F. Furthermore, the reaction times for the derivatization reaction of thiols with other conventional reagents [7,9,11–14,16,19,21] were longer than or as long as AcABD-F and TCAcABD-F: ThioGlo 3, NPM (5 min), DBD-F, mBBr, methyl 4-(6-methoxy-2-naphthyl)-4-oxo-2-butenoate (10 min), DBPM (30 min), SBD-F, and dansyl-aziridine (60 min). These results indicated that AcABD-F and TCAcABD-F are the most reactive reagents.

3.5. HPLC separation and detection of the derivatives with AcABD-F and TCAcABD-F

The chromatograms obtained from the thiols (GSH, Cys, Hcy, and Nac) derivatives with AcABD-F and TCAcABD-F are shown in Fig. 5A and 5B, respectively. The separations were completed within 15 min. No interfering peak was observed on the chromatogram. Fluorescence detection was performed at the maximum excitation and emission wavelengths in the mobile phase. The calibration curves for thiols (Cys, Hcy, and GSH) were linear over the range from 100 fmol to 100 pmol per

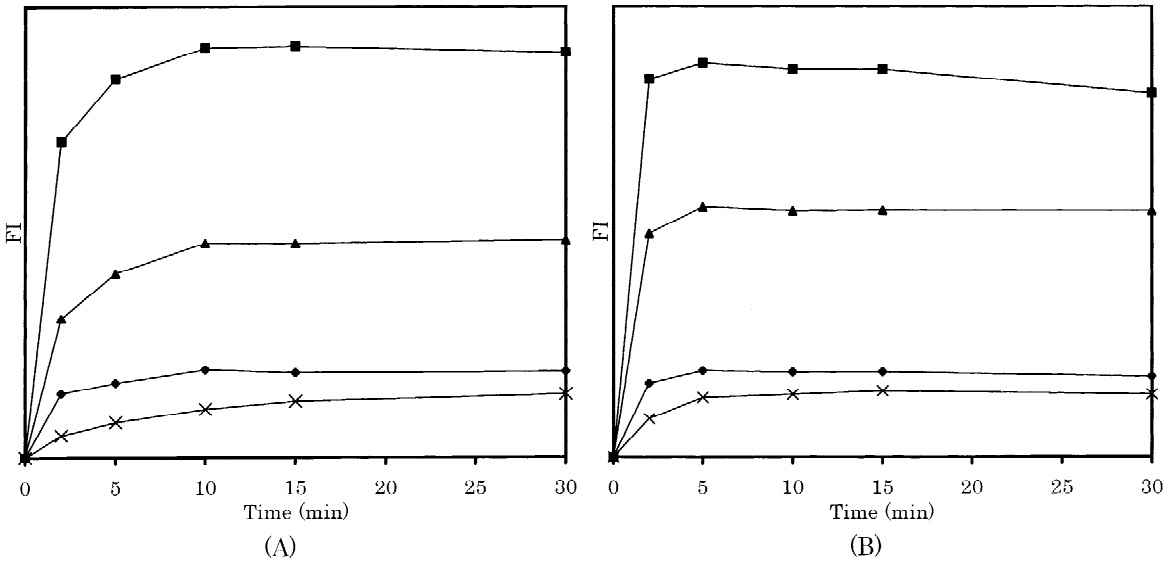

Fig. 4. Time courses of derivatization reaction of thiols with (A) AcABD-F and (B) TCAcABD-F. Symbols are Cys (♦), Hcy (■), GSH (▲), and Nac (×) (100 m*M*).

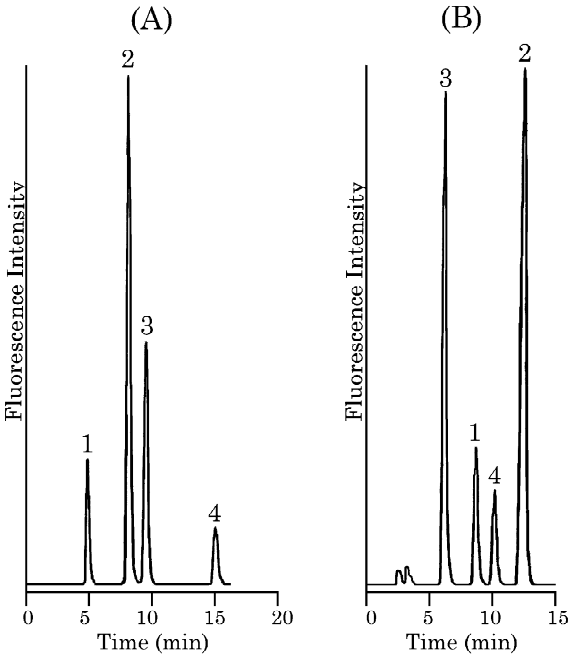

Fig. 5. Chromatograms of thiols derivatized with AcABD-F (A) and TCAcABD-F (B). (1) 45 pmol cysteine, (2) 45 pmol homocysteine, (3) 45 pmol glutathione, (4) 45 pmol *N*-acetylcysteine; flow-rate, 1.0 ml/min; detection, λ_{ex} 388 nm, λ_{em} 504 nm.

injection. The detection limits ($S/N = 3$) for the AcABD-derivatives were 63, 25, 41, and 155 fmol on column (28, 11, 18, and 69 n*M*), (Cys, Hcy, GSH, and Nac, respectively), and for the TCAcABD-derivatives were 82, 51, 45, and 164 fmol on column (36, 23, 20, and 73 n*M*), (Cys, Hcy, GSH, and Nac, respectively) which were lower than conventional reagents [8,9,11,12]: NAM (50 fmol), ThioGlo 3 (50 fmol), NPM (400 fmol), and mBBr (100 fmol).

4. Conclusion

New fluorogenic reagents for thiols, AcABD-F and TCAcABD-F were developed. These reagents were superior to other reagents with regard to their sensitivity and reactivity. Furthermore the derivatives of AcABD-F and TCAcABD-F ionized in neutral pH solution, which suggests that the derivatives of these reagents are highly soluble in water. These reagents seem to be useful for the derivatization of hydrophobic compounds bearing thiol groups, such as proteins.

Acknowledgements

We thank Dr. Chang-Kee Lim, MRC Bioanalytical Science Group, Birkbeck, University of London, for his valuable suggestions and discussion.

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