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4-(5′,6′-Dimethoxybenzothiazolyl)benzoyl Fluoride and 2-(5′,6′-Dimethoxybenzothiazolyl)benzenesulfonyl Chloride as Sensitive Fluorescence Derivatization Reagents for Amines in High-performance Liquid Chromatography

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4-(5′,6′-Dimethoxybenzothiazolyl)benzoyl fluoride (BHBT-COF) and 2-(5′,6′-dimethoxybenzothiazolyl)-benzenesulfonyl chloride (BHBT-SOCl) have been developed as highly sensitive and selective fluorescence derivatization reagents for primary and secondary aliphatic amines in HPLC. These reactivities were investigated using n-propylamine, n-heptylamine and *N*-methylhexylamine as model compounds to optimize the derivatization conditions. Both reagents readily reacted with the amines in basic media to give the corresponding fluorescent derivatives, which were separated isocratically by reversed-phase (C_{18}) liquid chromatography with aqueous methanol. The detection limits (signal-to-noise ratio = 3) of BHBT-COF for primary and secondary amines are approximately 3 and 30 fmol, respectively, and those of BHBT-SOCl are approximately 3 and 300 fmol, respectively, for an injection volume of 20 µl. Both reagents gave no fluorescent derivatives for aromatic amines.

Keywords: *4-(5′,6′-Dimethoxybenzothiazolyl)benzoyl fluoride; 2-(5′,6′-dimethoxybenzothiazolyl)benzenesulfonyl chloride; fluorescence derivatization reagents; amines; high-performance liquid chromatography*

Various fluorescence derivatization reagents having a halocarbonyl or halosulfonyl group have been reported for the determination of amines by HPLC, *e.g.*, 4-(*N*,*N*-dimethylaminosulfonyl)-7-(2-chloroformylpyrrolidin-1-yl)-2,1,3-benzoxazole,[1] 2-(9-anthroyl)ethyl chloroformate,[2] 9-fluorenylmethyl chloroformate,[3] phthalimidylbenzoyl chloride,[4] 2-(1-pyrenyl)ethyl chloroformate,[2] 6-methoxy-2-methylsulfonylquinoline-4-carbonyl chloride,[5] 7-dimethylaminocoumarin-3-carbonyl fluoride,[6] 1-dimethyl- and 1-di-*n*-butylaminonaphthalene-5-sulfonyl chloride,[7–9] 2-methylanilinonaphthalene-6-sulfonyl chloride[10] and 4-(*N*-phthalimidyl)benzenesulfonyl chloride.[11]

Recently, we developed 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-3-carbonyl chloride (DMEQ-COCl) as a highly sensitive and selective fluorescence derivatization reagent for amines.[12] It is one of the most sensitive reagents and has already been successfully applied to the determination of β-phenylethylamine in human plasma[13] and 1,2,3,4-tetrahydroisoquinoline in rat brain.[14]

In previous papers, we reported that 5,6-dimethoxy- 2-benzothiazole (BHBT), which is produced by the reaction between benzaldehyde and 2,2′-dithiobis(1-amino-4,5-dimethoxybenzene), gives an approximately three times more intense fluorescence than DMEQ and is excellent as a fluorophore.[15,16] In this work, we synthesized 4-(5′,6′-dimethoxybenzothiazolyl)benzoyl fluoride (BHBT-COF) and 2-(5′,6′-dimethoxybenzothiazolyl)benzenesulfonyl chloride (BHBT-SOCl) having a carbonyl fluoride and a benzenesulfonyl chloride, respectively, as the reactive groups toward amines. In order to investigate their reactivity with amines, n-propylamine, n-heptylamine and *N*-methylhexylamine were used as model primary and secondary amines; the reagents reacted with the amines in acetonitrile in the presence of quinuclidine or triethylamine to produce the corresponding fluorescent amides. The amides could be separated by reversed-phase HPLC with aqueous methanol. The structures of the amides and their fluorescence properties are also reported.

Experimental

Apparatus

Uncorrected fluorescence spectra and intensities were measured with a Hitachi (Tokyo, Japan) Model 650-60 spectrofluorimeter using a 10×10 mm silica cell; spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. ^{1}H NMR spectra were recorded on a Jeol (Tokyo, Japan) Model JNM-GX-400 spectrometer at 400 MHz using an approximately 1% m/v solution of [^{2}H]chloroform or [$^2H_6$]dimethyl sulfoxide containing tetramethylsilane as an internal standard (abbreviations used: s, singlet; d, doublet; t, triplet; q, quartet; and br, broad). Electron impact (EI) and fast atom bombardment (FAB) mass spectra were recorded on a Jeol Model DX-300 spectrometer. Uncorrected melting-points were determined on a Yazawa (Tokyo, Japan) micro melting-point apparatus.

Chemicals and Solutions

All chemicals were of analytical-reagent grade, unless stated otherwise. Water was de-ionized, distilled and further purified with a Milli-QII system (Japan Millipore, Tokyo, Japan). n-Propylamine, n-heptylamine and *N*-methylhexylamine were purchased from Wako (Osaka, Japan). Test solutions of the amines were prepared in acetonitrile. 2,2′-Dithiobis(1-amino-4,5-dimethoxybenzene) (DTAD) was prepared as described previously.[16].

For the preparation of BHBT-COF and BHBT-SOCl solutions, these reagents were first dissolved in 3-methoxyethanol and then diluted with acetonitrile to the desired concentrations. The reagent solutions were used within 24 h.

Synthesis of BHBT-COF and BHBT-SOCl

BHBT-COF and BHBT-SOCl were synthesized *via* BHBT-COOH and BHBT-SOH from DTAD, respectively (Fig. 1).

BHBT-COF

DTAD (2 g, 5.4 mmol) and 4-carboxybenzaldehyde (4 g, 2.6 mmol) were dissolved in 50 ml of ethanol containing 0.6 g of tri-n-butylphosphine and 0.8 m hydrochloric acid. The mixture was refluxed for 1 h with stirring and then cooled. The reaction mixture was concentrated to about 5 ml under reduced pressure. The resulting precipitates were filtered, washed with methanol–water (1 + 1 v/v) and dried under reduced pressure. The crude product was purified by column chromatography (20 × 3.5 cm id column; Japan Merck, Tokyo, Japan) on a silica gel 60 column (about 100 g, 70–230 mesh) with chloroform–methanol–water (20 + 2 + 0.1 v/v) as eluent. The main fraction was evaporated to dryness under reduced pressure and the residue was recrystallized from ethanol to give BHBT-COOH as pale yellow needles (yield 1.68 g, mp > 300 °C). ^{1}H NMR spectrum ([$^2H_6$]dimethylsulfoxide): δ_{1H} (ppm) 3.88, 3.89 (s each, 3H each, OCH_3 each); 7.64, 7.73 (s each, 1H each, aromatic proton in benzothiazole moiety); 8.08, 8.13 [d (J = 8.5 Hz) each, 2H each, aromatic protons in phenyl moiety]. Elemental analysis: calculated for $C_{16}H_{13}NO_4S$, C 60.94, H 4.16, N 4.44; found, C 60.72, H 4.30, N 4.63%. EI-MS: *m/z* 315 (M^+, base peak).

To a solution of BHBT-COOH (250 mg, 0.63 mmol) in 40 ml of dichloromethane were added 100 ml of pyridine and 60 ml of cyanuric fluoride. The mixture was stirred at room temperature for 1 h, then evaporated to dryness under reduced pressure. The residue was recrystallized from dichloromethane to give BHBT-COF as yellow needles (yield 168 mg, mp 228–230 °C). ^{1}H NMR spectrum ([^{2}H]chloroform): δ_{1H} (ppm) 3.99, 4.00 (s each, 3H each, OCH_3 each); 7.25, 7.58 (s each, 1H each, aromatic proton in benzothiazole moiety); 8.15, 8.18 [d (J = 8 Hz) each, 2H each, aromatic protons in phenyl moiety]. Elemental analysis: calculated for $C_{16}H_{12}FNO_3S$, C 60.56, H 3.81, N 4.41; found, C 60.64, H 3.82, N 4.42%. EI-MS: *m/z* 317 (M^+, base peak).

BHBT-COF was stable in the crystalline state for 1 year or longer when kept dry in the dark at room temperature.

BHBT-SOCl

To an aqueous solution of sodium 2-formylbenzenesulfonate (2.0 g, 9.6 mmol) occupying 30 ml was added DTAD solution (2.0 g, 5.4 mmol) in 50 ml of ethanol containing 0.1 mg of tri-n-butylphosphine and 2 m hydrochloric acid. The mixture was refluxed for 1 h. The precipitates were filtered, washed with water and dried under reduced pressure. The crude product was recrystallized from ethanol to give BHBT-SOH as colorless needles (yield 800 mg, mp > 300 °C). ^{1}H NMR spectrum ([^{2}H]chloroform): δ_{1H} (ppm) 4.00, 4.01 (s each, 3H each, OCH_3 each); 7.26, 7.67 (s each, 1H each, aromatic proton in benzothiazole moiety); 7.83, 8.30 [d (J = 8 Hz) each, 2H each, aromatic protons in phenyl moiety]. Elemental analysis: calculated for $C_{15}H_{13}NO_5S_2$, C 51.22, H 3.73, N 3.99; found, C 52.12, H 3.74, N 4.00%. EI-MS: *m/z* 351 (M^+, base peak).

BHBT-SOH (600 mg, 1.4 mmol) was dissolved in 20 ml of freshly distilled thionyl chloride and the mixture was heated at reflux for 1 h and then cooled. The precipitates formed on adding about 50 ml of light petroleum (bp 30–60 °C) were collected by filtration and were recrystallized from benzene–petroleum (1 + 1 v/v) to give BHBT-SOCl as pale yellow needles (yield 500 mg, mp > 300 °C). ^{1}H NMR spectrum ([^{2}H]chloroform): δ_{1H} (ppm) 4.00, 4.01 (s each, 3H each, OCH_3 each); 7.26, 7.67 (s each, 1H each, aromatic proton in benzothiazole moiety); 7.83, 8.30 [d (J = 8 Hz) each, 2H each, aromatic protons in phenyl moiety]. Elemental analysis: calculated for $C_{15}H_{12}NO_4S_2Cl$, C 48.72, H 3.27, N 3.79; found, C 49.50, H 3.28, N 3.80%. EI-MS: *m/z* 369 (M^+, base peak).

In the crystalline state, BHBT-SOCl remained stable for 1 year or longer when kept dry in the dark at room temperature.

Isolation of the Reaction Products of n-Propylamine with BHBT-COF and BHBT-SOCl (Fig. 2)

BHBT-COF (200 mg), n-propylamine (100 mg) and quinuclidine (100 mg) were dissolved in acetonitrile (30 ml) and the mixture was allowed to stand at room temperature for 1 h. The mixture was evaporated to dryness under reduced pressure. The residue dissolved in a small amount of chloroform was chromatographed on a silica gel 60 column (25 × 2.5 cm id, 75 g) with the same solvent. The main fraction was evaporated to dryness under reduced pressure and the residue was recrystallized from ethyl acetate to give product **I** as colorless needles (yield 150 mg, mp 221–223 °C). ^{1}H NMR spectrum ([^{2}H]chloroform): δ_{1H} (ppm) 1.02 (t, 3H, CH_3); 1.67, 1.88 (q each, 2H each); 3.98, 3.99 (s each, 3H each, OCH_3 each); 6.15 (br s, 1H, NH); 7.26, 7.57 (s each, 1H each, aromatic proton in benzothiazole moiety); 7.86, 8.10 [d (J = 8 Hz) each, 2H each, aromatic protons in phenyl moiety]. Elemental analysis: calculated for $C_{19}H_{20}N_2O_3S$, C 64.02, H 5.66, N 7.86; found, C 64.09, H 5.67, N 7.88%. FAB-MS: *m/z* 357 ($[M + 1]^+$, base peak).

BHBT-SOCl (200 mg), n-propylamine (100 mg) and triethylamine (100 mg) were dissolved in acetonitrile (20 ml) and the mixture was allowed to stand at room temperature for 30 min. The mixture was evaporated to dryness under reduced pressure. The residue dissolved in a small amount of chloroform was chromatographed on a silica gel 60 column (25 × 2.5 cm id, 75 g) with the same solvent. The main fraction was evaporated to dryness under reduced pressure and the residue was recrystallized from ethyl acetate–n-hexane (1 + 1 v/v) to afford product **II** as colorless needles (yield 140 mg, mp 160–162 °C). ^{1}H NMR spectrum ([^{2}H]chloroform): δ_{1H} (ppm) 0.96 (t, 3H, CH_3); 1.63, 1.82 (q each, 2H each); 2.98 (br s, 1H, NH); 3.98, 4.00 (s each, 3H each, OCH_3 each); 7.26, 7.45 (s each, 1H each,

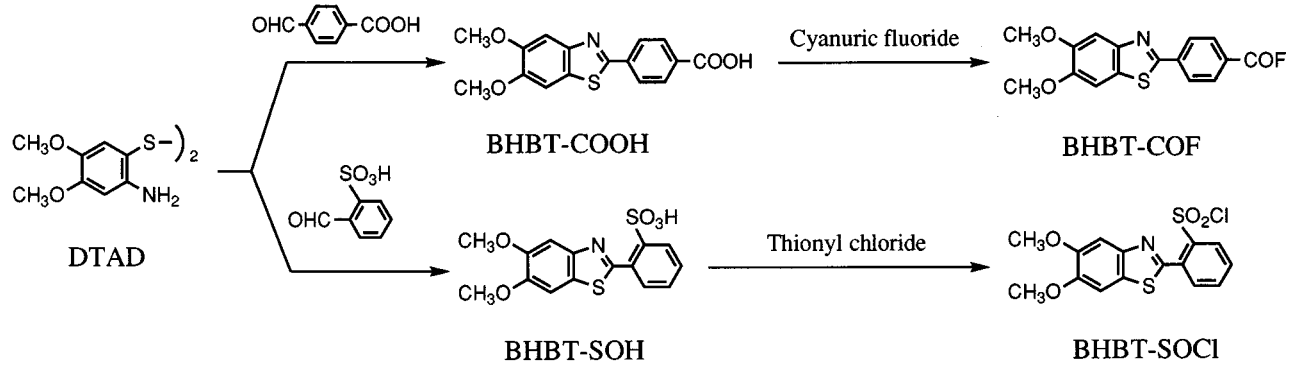


Fig. 1 Synthesis of BHBT-COF and BHBT-SOCl.

aromatic proton in benzothiazole moiety); 7.75, 8.22 [d (J = 8 Hz) each, 2H each, aromatic protons in phenyl moiety]. Elemental analysis: calculated for $C_{18}H_{20}N_2O_4S_2$, C 55.08, H 5.14, N 7.14; found, C 56.02, H 5.16, N 7.15%. FAB-MS; m/z 393 ($[M + 1]^+$, base peak).

Derivatization Procedures with BHBT-COF and BHBT-SOCl

BHBT-COF

To 100 μl of a test solution of amines in acetonitrile were added 100 μl of 10 mmol l^{-1} quinuclidine in acetonitrile and 100 μl of 3 mmol l^{-1} BHBT-COF in acetonitrile. The mixture was allowed to stand at 37 °C for 20 min, then a portion (20 μl) was injected into the chromatograph. To prepare the reagent blank, 100 μl of acetonitrile in place of the test solution were subjected to the same procedure.

BHBT-SOCl

To 100 μl of a test solution of amines in acetonitrile were added 100 μl of 10 mmol l^{-1} triethylamine in acetonitrile and 100 μl of 1 mmol l^{-1} BHBT-SOCl in acetonitrile. The mixture was allowed to stand at room temperature for about 2–3 min, then a portion (20 μl) was injected into the chromatograph. To prepare the reagent blank, 100 μl of acetonitrile in place of the test solution were subjected to the same procedure.

HPLC Apparatus and Conditions

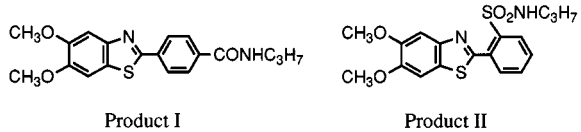
A Hitachi Model 655A liquid chromatograph equipped with a Rheodyne (Cotati, CA, USA) Model 7125 syringe-loading sample injection valve (20 μl loop) and a Shimadzu (Kyoto, Japan) RF-535 spectrofluorimeter fitted with a 12 μl flow-cell was used. The fluorescence intensity was monitored with excitation at 350 nm and emission at 450 nm for BHBT-COF and with excitation at 330 nm and emission at 450 nm for BHBT-SOCl. A TSK gel ODS- 120T (Tosoh, Tokyo, Japan) column (250 × 4.6 mm id, particle size 5 μm) was used. The column temperature was ambient (25 + 2 °C). The mobile phase was methanol–water (70 + 30 v/v), delivered at flow rate of 1.0 ml min^{-1}.

Results and Discussion

Fluorescent Products of the Reaction Between Amines and BHBT-COF or BHBT-SOCl

The reaction products obtained in the determination of amines were examined by using n-propylamine. The reaction products of BHBT-COF and BHBT-SOCl with n-propylamine were found to be BHBT-carbonyl amide and BHBT-sulfonyl amide, respectively. These amides were confirmed as products **I** and **II** (Fig. 2) from the elemental analysis, MS and ^{1}H NMR data.

The fluorescence properties (excitation and emission spectra and intensities) of their products were measured in various solvents (Table 1). The excitation and emission spectra of each product were almost identical in shape and the maxima were independent of the solvents used. Products **I** and **II** provided the most intense fluorescence in methanol and acetonitrile. However, the fluorescence intensity from product **II** was about 10–20% of that from product **I**.



Product I Product II

Fig. 2 Reaction products of n-propylamine with BHBT-COF and BHBT-SOCl.

Chromatographic Conditions

The separation of the fluorescent derivatives of BHBT-COF with n-propylamine, n-heptylamine, and *N*-methylhexylamine was studied on a reversed-phase column (TSK gel ODS-120 T) with aqueous acetonitrile. At methanol concentrations > 80% v/v, the peak for n-propylamine was not fully resolved from that given by the reagent blank, whereas methanol concentrations < 60 % v/v caused a delay in elution with peak broadening. Optimum separation was obtained with 75% v/v methanol in water. When aqueous acetonitrile was used as the mobile phase, the half-widths of the peaks were about doubled. Also, similar results were obtained for the separation of BHBT-SOCl derivatives of the amines.

Fig. 3(A) and (B) are typical chromatograms of BHBT-carbonyl amide and BHBT-sulfonyl amide derivatives of the three amines. The individual compounds gave a single peak.

Derivatization Conditions

The derivatization conditions were examined by using a mixture of n-propylamine, n-heptylamine and *N*-methylhexylamine (1 × 10^{-6} mol l^{-1} each).

Table 1 Excitation and emission maxima and relative fluorescence intensities (RFI) from products **I** and **II** in various solvents

	Product **I**			Product **II**		
Solvent	λ_{ex}/nm	λ_{em}/nm	RFI[a]	λ_{ex}/nm	λ_{em}/nm	RFI[a]
Water	348	465	73	332	462	8
Methanol	351	450	100	333	450	18
Acetonitrile	351	450	100	332	448	18
Chloroform	351	440	98	334	436	17
Ethyl acetate	350	450	94	334	440	15
Benzene	356	450	79	335	435	13

[a] The fluorescence intensity of product **I** in methanol was taken as 100.

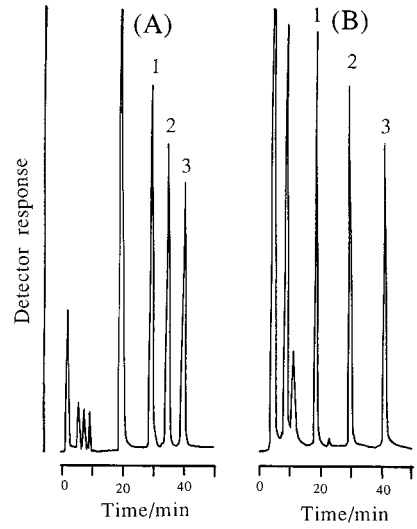


Fig. 3 Chromatograms of (A) BHBT-carbonyl amide and (B) BHBT-sulfonyl amide derivatives of n-propylamine, n-heptylamine and *N*-methylhexylamine. Portions (100 μl) of a standard mixture of the three amines (1 × 10^{-6} mol l^{-1} each) were treated according to the described procedures and subjected to HPLC. Peaks: 1, n-propylamine; 2, n-heptylamine; 3, *N*-methylhexylamine; and others, reagent blank.

Acetonitrile as a solvent for the derivatization reaction provided the most intense peaks for both reagents; acetone and benzene gave less intense peaks (about 20 and 10%, respectively, of those obtained in acetonitrile). On the other hand, the fluorescence reaction was very limited in chloroform, cyclohexane, dimethyl sulfoxide and *N*,*N*-dimethylformamide. Acetonitrile was therefore chosen for the recommended procedures.

Concentrations of BHBT-COF and BHBT-SOCl of 2.5–6.0 and 0.8–6.0 mmol l^{-1}, respectively, in their reagent solutions provided almost maximum and constant peak heights from the three amines; 3.0 and 1.0 mmol l^{-1} solutions for BHBT-COF and BHBT-SOCl, respectively, were adopted in the recommended procedures.

It is well known that quinuclidine, pyridine and triethylamine facilitate the derivatization of amines with reagents having a halocarbonyl or halosulfonyl group. Of these bases, quinuclidine (7–20 mmol l^{-1} in the solution) for BHBT-COF and triethylamine (5–20 mmol l^{-1} in the solution) for BHBT-SOCl provided the most intense peaks for the three amines; 10 mmol l^{-1} quinuclidine and 10 mmol l^{-1} triethylamine were employed in the procedures. In the absence of these bases, the peak heights were about 50% of those obtained with these bases.

The derivatization reactions of the amines with BHBT-COF and BHBT-SOCl proceeded fairly rapidly even at 0 °C (Fig. 4). In the case of BHBT-COF, higher temperatures allowed the fluorescent peak heights to develop more rapidly. However, the peak heights were decreased by longer heating and higher temperature. Hence a temperature of 37 °C and a reaction time of 10 min were selected for the derivatization of the amines with BHBT-COF. On the other hand, with BHBT-SOCl the maximum and constant peak heights were obtained at 1–30 min, irrespective of the temperature (20–80 °C). Therefore, standing for about 2–3 min at room temperature was used in the procedure.

All the amide derivatives of BHBT-COF and BHBT-SOCl in the final mixtures were stable for at least 10 h in daylight at room temperature. The yields of the fluorescent amide derivatives from BHBT-COF and BHBT-SOCl under the conditions employed were found to be 92.6 and 93.8%, respectively, by comparing the value of peak height for n-propylamine with those of products I and II.

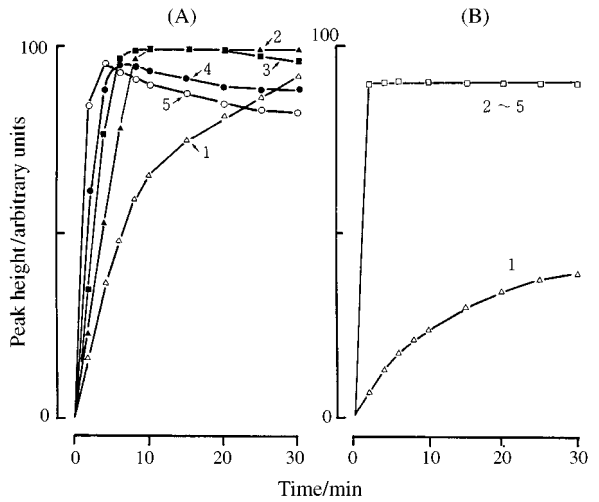


Fig. 4 Effect of reaction time and temperature on the peak height of n-heptylamine. Portions (100 μl) of n-heptylamine (1 nmol ml^{-1}) were treated according to the procedure, but at different temperatures. Temperature: 1, 0 °C, 2, 20 °C; 3, 37 °C; 4, 50 °C; and 5, 80 °C. (A) BHBT-COF; and (B) BHBT-SOCl.

Precision, Calibration Graph and Detection Limit

For both reagents, the calibration graphs were linear over the concentration range 10 fmol–100 pmol in a 20 μl injection volume for all the amines examined. The precision was established by repeating 10 analyses concurrently on 1 and 10 pmol each per 20 μl. The RSDs for both reagents did not exceed 2.3% for all the amines. The detection limits (signal-to-noise ratio = 3) obtained with BHBT-COF were 1.4 (n-propylamine), 1.7 (n-heptylamine) and 2.0 fmol (*N*-methylhexylamine), and those with BHBT-SOCl were 10.2 (n-propylamine), 11.9 (n-heptylamine) and 13.1 (*N*-methylhexylamine) fmol per 20 μl injection volume. The sensitivity obtained with BHBT-COF was higher than that with DMEQ-COCl, which is one of the most sensitive reagents for amines. BHBT-SOCl gave almost the same sensitivity as DMEQ-COCl.[12–14]

Reaction of Other Substances with BHBT-COF and BHBT-SOCl

Many amines reacted with both reagents under the derivatization conditions; n-butylamine, n-amylamine, n-hexylamine, n-octylamine, n-nonylamine, cyclohexylamine, benzylamine, 4-methylbenzylamine, di-n-propylamine, di-n-butylamine, diethylamine, *N*-ethylbenzylamine, dibenzylamine, tyramine, histamine and 2-phenylethylamine. All these amines gave single fluorescent peaks in the chromatograms. Seventeen l-α-amino acids and aromatic amines (aniline and *o*-toluidine) did not give fluorescent peaks under the derivatization and HPLC conditions used.

No other biologically important substance examined fluoresced under the recommended conditions at a concentration of 20 nmol ml^{-1}. The compounds tested were methanol, ethanol, n-butanol, alloxan, ascorbic acid, glutathione, thiamine, citrulline, allantoin, uric acid, urea, bilirubin, acetone, cyclohexane, 4-methylcyclohexane, acetylacetone, acetophenone, benzil, lactic acid, 3-hydroxybutyric acid, acetoacetic acid, homogentisic acid, pyruvic acid, phenylpyruvic acid, *N*-acetylneuraminic acid, inositol, d-xylose, d-glucose, d-fructose, d-mannose, d-maltose, d-lactose, epiandrosterone, dehydroepiandrosterone, cortisone, cholesterol and methylglyoxal. These results suggested that the present derivatization method is usefully selective for amines.

Conclusion

The proposed method permits the highly sensitive and selective determination of primary and secondary aliphatic amines. The reaction is completed very rapidly at room temperature and the resulting fluorescent derivatives are stable for at least 10 h. The proposed reagents can be applied to the determination of biogenic amines in body fluids; further studies are in progress.

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