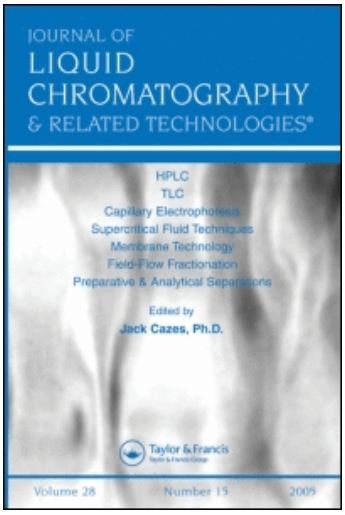

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5,6-Dimethoxy-2-(4'-Hydrazinocarbonylphenyl)benzothlazole as a Highly Sensitive and Stable Fluorescence Derivatization Reagent for Carboxylic Acids in High Performance Liquid Chromatography

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5,6-DIMETHOXY-2-(4'-HYDRAZINOCARBONYL-PHENYL)BENZOTHIAZOLE AS A HIGHLY SENSITIVE AND STABLE FLUORESCENCE DERIVATIZATION REAGENT FOR CARBOXYLIC ACIDS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

5,6-Dimethoxy-2-(4'-hydrazinocarbonylphenyl)benzothiazole (BHBT-hydrazide) was found to be a highly sensitive and stable fluorescence derivatization reagent for carboxylic acids in liquid chromatography. Its reactivity was investigated for various $C_{18:0}$ - $C_{22:6}$ saturated and unsaturated fatty acids. The reagent readily reacted with the fatty acids in aqueous solution in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 37℃ to produce the corresponding fluorescent derivatives. The derivatives were separated on a reversed-phase column, TSK-gel ODS 120T, with gradient elution using 40 - 100 % (v/v) aqueous acetonitrile, and were detected spectrofluorimetrically at 447 nm with excitation of 365 nm. Calibration curves were linear over the range 10 fmol - 5.0 pmol per 20-μl injection (r=0.992 - 0.999). The relative standard deviations for ten replicated determinations did not exceed 2.0 % for any of the fatty acids (1.0 nmol/ml). The detection limits (signal-to-noise ratio=3) for the acids were 1 - 2 fmol

for an injection volumn of 20 μl. Further, the BHBT derivative of stearic acid was synthesized to examine the fluorescence properties.

INTRODUCTION

Various biogenic carboxylic acids occur in extremely small amounts in biological samples, and play very important roles. In addition, many drugs with carboxylic acids, which have strong efficacy against diseases and are administered at extremely small doses, have been developed. Therefore, a sensitive and selective high-performance liquid chromatographic (HPLC) method is required for the determination of the biogenic carboxylic acids and drugs.

For the purpose, numerous fluorescence derivatization reagents have been developed for the determination of carboxylic acids by HPLC: *e.g.* 4-bromomethyl-7-methoxy- (1-3), 4-hydroxymethyl-7-methoxy- (4), 4-diazomethyl-7-methoxy- (5), 4-bromomethyl-7-acethoxy- (6), 4-bromomethyl-6,7-dimethoxycoumarins (7), *N,N'*-dicyclohexyl-*O*-(7-methoxycoumarin-4-yl)methylisourea (8), 8-(chloromethyl)- (9), 9-(hydroxymethyl)- (10) and 9-aminophenanthrenes (11), 9-anthryldiazomethane (12,13), 9,10-diaminophenanthrene (14), *p*-(9-anthroyl)phenacyl bromide (15), 1-aminoethyl-4-dimethylaminonaphthalene (16), 1-bromoacetylpyrene (17), monodansyl cadaverine (18,19), 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate (20), 4-substituted 7-aminoalkylamino-2,1,3-benzoxadiazoles (21,22), 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (23), 9-bromomethylacridine (24), *N*-(9-acridinyl)bromoacetamide (25), 1-pyrenyldiazomethane (26) and 3-bromomethyl-6,7-dimethoxy- (27) and 3-bromomethyl-6,7-methylenedioxy-1-methyl-2(1*H*)quinoxalinones (28). However, these reagents generally require almost dried aprotic solvents, higher temperatures and/or prolonged heating in the derivatization reaction.

Most of biogenic substances and drugs having carboxylic acids are polor molecules, and occasionally labile against heat and light. Accordingly, a stable and highly sensitive reagent, which reacts with carboxylic acids in aqueous solution under mild reaction conditions, is required for the determination of their substances. Recently, in order to overcome the problems, some sensitve and selective fluorescence derivatization reagents having a hydrazinocarbonyl group as the reactive

group towards carboxylic acids have been reported; *viz* 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-hydrazide) (29), 4-(5,6-dimethoxy-2-benzimidazoyl)benzohydrazide (30) and 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (31). The reagents react with carboxylic acids in aqueous solution in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide under moderate derivatization conditions. Of these reagents, DMEQ-hydrazide has been already successfully applied for the direct determination of plasma free fatty acids (32) and urinary steroid glucuronides (33).

In previous research, we reported that 5,6-dimethoxy-2-benzothiazole, which is produced by the reaction between benzaldehyde and 2,2'-dithiobis-(1-amino-4,5-dimethoxybenzene), gives a more intense fluorescence than DMEQ and is excellent as a fluorophore (34). Hence, we newly synthesized 5,6-dimethoxy-2-(4'-hydrazinocarbonylphenyl)benzothiazole (BHBT-hydrazide), which has a novel 5,6-dimethoxy-2-benzothiazole moiety, as a fluorescence derivatization reagent for carboxylic acids. The reagent was demonstrated to be very useful for the highly sensitive determination of linear saturated and unsaturated fatty acids in the fluorimetric HPLC.

Moreover, the BHBT derivative of stearic acid was isolated to examine the fluorescence properties.

EXPERIMENTAL

Apparatus

Uncorrected fluorescence spectra and intensities were measured with a Hitachi (Tokyo, Japan) Model 650-60 spectrofluorimeter in 10 x 10 mm quartz cells; the spectral bandwidths of 10 nm were used for both the excitation and emission monochromators. Electron impact (EI) and fast atom bombardment (FAB) mass spectra (MS) were recorded with a Jeol (Tokyo, Japan) DX-300 spectrometer. ^{1}H-nuclear magnetic resonance (NMR) spectra were obtained with a Jeol JNM-GX400 spectrometer at 400 MHz using a ca. 1 % (w/v) solution of chloroform-d_1 or dimethyl-d_6 sulphoxide containing tetramethylsilane as an internal standard (abbreviations used : s, singlet; d, doublet; t, triplet; m, multiplet). Uncorrected melting temperatures were measured on a Yanaco (Tokyo, Japan) micro melting-point apparatus.

Chemicals and solutions

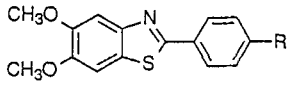
All chemicals were of analytical-reagent grade, unless stated otherwise. Deionized distilled water was used. Caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), myristoleic ($C_{14:1}$), palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), margaric ($C_{17:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), linolenic ($C_{18:3}$), arachidic ($C_{20:0}$), arachidonic ($C_{20:4}$) and docosahexaenoic ($C_{22:6}$) acids were purchased from Sigma (St. Louis, MO, USA). Stock solutions of $C_{8:0}$ - $C_{22:6}$ acids (1×10^{-4} M) were prepared in *N,N*-dimethylformamide and diluted further with water to give the required concentrations. 2,2'-Dithiobis(1-amino-4,5-dimethoxybenzene) (DTAD) was prepared as described previously (34).

Synthesis of BHBT-hydrazide

BHBT was synthesized via compounds I-II from DTAD in satisfactory yields by the following method (Fig. 1).

Compounds I and II

To a solution of 4-carboxylbenzaldehyde [1.2 g (8 mmol)/20 ml methanol] was added DTAD solution [3.7g (10 mmol)/40 ml methanol containing 1.2 g of tri-*n*-buthylphosphine and 0.8 M disodium hydrogen phosphite]. The mixture was allowed to stand at 37℃ for 2 h with stirring. The precipitates were filtered, washed with methanol - water (7:3, v/v) and dried under reduced pressure. The resulting crude product (compound I) dissolved in absolute methanol (50 ml) was treated with ethereal diazomethane solution prepared by the established method (35). The reaction mixture was evaporated to dryness under reduced pressure. The residue was subjected to chromatography on a silica gel 60 (ca. 130 g. 70-230 mesh; Japan Merck, Tokyo) column (25 x 3.5 cm i.d.) with *n*-hexane - ethylacetate - chloroform (2:1:1, v/v) as eluent. The main fraction was collected and the solvent was removed under reduced pressure to give compound II (800 mg, 30 %) as pale yellow needles, m.p. 223 - 224℃. ^{1}H NMR spectra (chloroform-d_1); δ 3.96, 3.98, 3.99 (s each, 3H each, OCH_3 each); 7.31, 7.56 (s each. 1H each, aromatic proton in beozothiazole moiety]; 8.08, 8.12 ppm [d (J=8 Hz) each, 2H each, aromatic proton in phenyl moiety]. Elemental analysis, calculated for $C_{17}H_{15}NO_4S$, C 61.99, H 4.59, N 4.25; found, C 62.11, H 4.66, N 4.41 %. EI-MS, *m/z*=329 (M^+, base peak), 314 ($M^+ - CH_3$), 286 ($M^+ - CO - CH_3$).



	R
Compound I	COOH
Compound II	$COOCH_3$
BHBT-hydrazide	$CONHNH_2$
BHBT-ST	$CONHNHCO(CH_2)_{16}CH_3$

Fig. 1. Compound, reagent and product structures.

BHBT-hydrazide

To a solution of compound II (500 mg, 15 mmol) in ethanol (50 ml) was added 100 ml of aqueous 45 % hydrazine hydrate, and the mixture was refluxed for ca. 60 min. The resulting precipitate was collected and recrystallized from ethanol to give BHBT-hydrazide (400 mg, 80 %) as colorless needles, m.p. 252 - 254℃. ^{1}H NMR (dimethyl-d_6 sulphoxide), δ 3.87, 3.89 (s each, 3H each, OCH_3 each), 4.55 (s, 2H, NH_2), 7.62, 7.71 (s each, 1H each, aromatic proton in benzothiazole moiety), 7.98, 8.08 [d (J=8 Hz) each, 2H each, aromatic proton in phenyl moiety], 9.90 ppm (s, 1H, NH). Elemental analysis: calculated for $C_{16}H_{15}N_3O_3S$, C 58.35, H 4.59, N 12.76; found, C 58.03, H 4.63, N 12.69 %. El-MS, *m/z*=329 (M^+, base peak), 314 ($M^+ - CH_3$), 286 ($M^+ - CO - CH_3$).

BHBT-hydrazide was stable in the crystalline state for a year or longer even in daylight. The reagent dissolved in *N,N*-dimethylformamide could be used for at least 2 weeks.

Isolation of the reaction product of stearic acid and BHBT-hydrazide

BHBT-hydrazide (200 mg), stearic acid (180 mg) and EDC (95 mg) were dissolved in pyidine - ethanol (2:8, v/v) (50 ml) and the mixture was allowed to stand at 37℃ for 1 h. The mixture was evaporated to dryness under reduced pressure. The residue in a small amount of ethylacetate, was chromatographed on a silica gel 60 colunm (25 x 2.5 cm i.d., 75 g, 70-230 mesh) with the same solvent. The main fraction was evaporated to dryness and the residue was recrystallized from ethanol to give 160 mg (44 %) of BHBT-ST (Fig. l) as colorless needles, m.p. 230

- 232℃. ^{1}H NMR (dimethyl-d_6 sulphoxide), δ 0.85 [t (J=7 Hz), 3H, CH_3], 1.24 - 3.54 (m, 32H, $C_{16}H_{32}$), 3.88, 3.89 (s each, 3H each, OCH_3 each), 5.6 (broad, 1H, NH), 7.63, 7.73 (s each, 1H each, aromatic proton in benzothiazole moiety), 8.02, 8.03 [d (J=8 Hz) each, 2H each, aromatic protons in phenyl moiety], 9.03 ppm (broad, 1H, NH). Elemental analysis: calculated for $C_{34}H_{49}N_3O_4S$, C 68.32, H 8.41, N 6.91; found, C 68.54, H 8.29, N 7.05 %. FAB-MS, *m/z*=596 (MH^+), 298 (MH^+ - $C_{18}H_{36}N_2O_2$).

HPLC apparatus and conditions

A Hitachi 655A liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injection valve (20-μl loop) and a Shimadzu (Kyoto, Japan) RF-535 fluorescence spectromonitor fitted with a 12-μl flow-cell was used. The fluorescence spectrometer was operated at an excitation wavelength of 365 nm and an emission wavelength of 447 nm. A TSK gel ODS 120T (Tosoh, Tokyo, Japan) column (250 x 4.6 mm i.d.; particle size, 5 μm) was used. The column temperature was ambient (20 - 27℃). For the separation of the BHBT derivatives of fatty acids, a gradient elution with aqueous 40-100 % (v/v) acetonitrile was carried out by using a Hitachi 833A solvent gradient device. The flow-rate was 1.0 ml/min.

Derivatization procedure

To 100 μl of a test solution of fatty acids in water (or 20-times water diluted serum) were added 100 μl each of 1 M EDC and 20 % (v/v) pyridine (both in methanol) and 100 μl of 15 mM reagent in *N,N*-dimethylformamide. The mixture was allowed to stand at 37℃ for 20 min, and then a portion (20 μl) was injected into the chromatograph. To prepare the reagent blank, 100 μl of methanol in place of 100 μl of the test solution were subjected to the same procedure.

RESULTS AND DISCUSSION

Product of the reaction of stearic acid with BHBT-hydrazide and its fluorescence properties

The fluorescent reaction products obtained in the determination of carboxylic acids were examined by using stearic acid. As carbonylhydrazino groups react with carboxylic acid compounds in the presence of EDC to give acid hydrazides (29-31),

the reaction product from stearic acid should be the corresponding BHBT-carboxylic acid hydrazide derivative. The derivative was confirmed as BHBT-ST (in Fig. 1) by the elemental analysis data and by the FAB-MS and ^{1}H NMR spectral data.

The fluorescence properties of BHBT-ST in methanol, acetonitrile, water and their mixtures, which have been widely used as mobile phase in reversed-phase chromatography, were examined to find a suitable mobile phase for the HPLC separation of the BHBT derivatives of fatty acids ($C_{8:0}$ - $C_{22:6}$).

The fluorescence excitation (maximum, 365 nm) and emission (maximum, 447 nm) spectra of BHBT-ST in methanol were almost identical with those in water and acetonitrile. The maxima in aqueous methanol and acetonitrile were independent of the concentration of water. The maximum and constant intensity was obtained at wide ranges in aqueous acetonitrile [0-50 % (v/v)] in comparison with aqueous methanol [0-10 % (v/v)] (Fig. 2). These results suggest that aqueous acetonitrile is suitable as a mobile phase in reversed-phase chromatography of BHBT derivatives of fatty acids with gradient elution.

Derivatization conditions

The conditions were examined using a mixture of the fatty acids (1.0 nmol/ml each).

BHBT-hydrazide was found to dissolve easily in *N,N*-dimethylformamide and dimethylsulphoxide and slightly in acetonitrile, methanol, ethanol, 2-methoxyethanol and tetrahydrofurane, but not in water and acetone. The former seven solvents affected the reactivity between BHBT-hydrazide and fatty acids. Since *N,N*-dimethylformamide gave the most intense peak heights (Table 1), the reagent solution was therefore prepared in *N,N*-dimethylformamide.

The most intense peaks were obtained at concentrations greater than ca. 10 mM of the reagent solution for all the fatty acids; 15 mM was used in the procedure.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and pyridine were used to facilitate the derivatization of fatty acids with BHBT-hydrazide. Maximum and constant peak heights could be attained at EDC concentrations in the solution in the range of 0.8 - 1.3 M; 1.0 M was selected as optimum. The peak heights for the acids were maximum and constant at concentrations of pyridine higher than 10 % (v/v); 20 % was employed.

The derivatization reaction of fatty acids with BHBT-hydrazide apparently occurred even at 0℃; higher temperature allowed the fluorescence to develop more

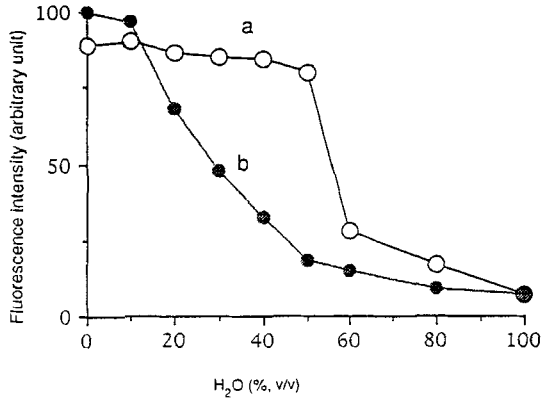


Fig. 2. Effect of water concentration in (a) aqueous acetonitrile and (b) aqueous methanol on the fluorescence intensity of BHBT-ST (1.0 nmol/ml). The fluorescence intensity was measured at the excitation and emission maxima.

TABLE 1

Effect of reaction solvent on the peak height

Solvent	Relative peak height[a)]
N,N-Dimethylformamide	100.0
Dimethylsulphoxide	94.5
Acetonitrile	76.4
Methanol	54.2
Ethanol	53.1
2-Methoxyethanol	60.4
Tetrahydrofurane	15.3

a) The peak height obtained with *N,N*-dimethylformamide was taken as 100.0.

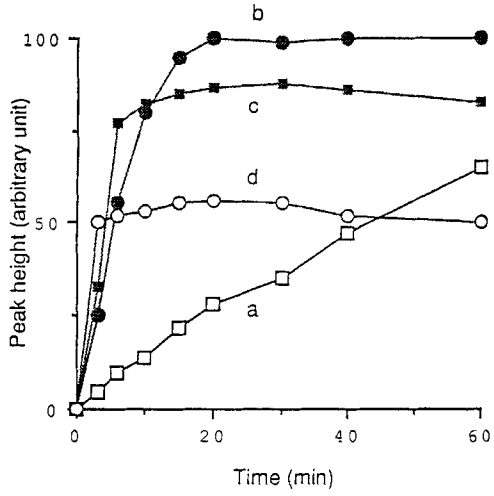


Fig. 3. Effect of reaction time and temperature on the peak height. A portion (100 μl) of a standard solution of $C_{16:0}$ acid (1.0 nmol/ml) was treated according to the described procedure. Temperature : a=0℃; b=37℃; c=50℃; d=80℃.

rapidly (Fig. 3). However, temperatures higher than 50℃ caused reduction of the fluorescence, probably because of decomposition of the produced derivatives. At 37 ℃, the peak heights for all the fatty acids were almost maximum after standing for 20 min. Hence, the solution was allowed to stand at 37℃ for 20 min in the procedure. The BHBT derivatives in the final mixture were stable for at least 24 h in the daylight at room temperature. The yield of the fluorescent derivative from stearic acid under the conditions employed was found to be 74.2 % by comparing the value of the peak height for stearic acid with that of BHBT-ST.

The similar optimal derivatization conditions were obtained when 2.0 pmol - 1.0 nmol/ml solutions of fatty acids were used.

Separation of BHBT derivatives of fatty acids

The good separation of BHBT derivatives of fifteen ($C_{8:0}$ - $C_{22:6}$) fatty acids was achieved on a reversed-phase column, TSK gel 120T, by gradient elution with acetonitrile between 40 and 100 % (v/v) in the mobile phase. However, the peaks for $C_{18:2}$ and $C_{20:4}$ acids could not be resolved successfully under any HPLC

conditions tested. The peak for $C_{20:1}$ acid (t_R=91.5 min) was slightly separated from the peak for $C_{18:0}$ acid (t_R=90.1 min). A typical chromatogram obtained with a standard solution of the fatty acids is shown in Fig. 4. The individual fatty acids each gave a single peak in the chromatogram. The change in the acetonitrile concentration had no effect on the fluorescence excitation and emission maximum wavelengths of the BHBT derivatives of all the fatty acids; the spectra were virtually identical with those of BHBT-ST.

It appears from Fig. 4 that different fatty acids have different peak responses. This might be due to the differences in the yields of the fluorescence derivatives from fatty acids and/or the quantum yields of the derivatives.

Precision, calibration graph and detection limit

The precision was established by repeated determinations using a standard mixture of fatty acids (1.0 nmol/ml each). The relative standard deviations did not exceed 2.0 % for any of the fatty acids examined (n=10 each instance).

The relationships between the peak heights and the amounts of the individual fatty acids were linear from 10 fmol to at least 5.0 pmol per injection volume (20 µl). The linear correlation coefficients were 0.992 or better for all the fatty acids.

The detection limits for all the fatty acids were 1 - 2 fmol on-column (signal-to-noise ratio=3). The method with BHBT-hydrazide was more sensitive 3 - 4 times than that with DMEQ-hydrazide (30), which is one of the most sensitive HPLC method.

Reaction of BHBT-hydrazide with compounds other than linear saturated and unsaturated $C_{8:0}$ - $C_{22:6}$ fatty acids

Hydroxycarboxylic acids (lactic and malic), dicarboxylic acids (oxalic, malonic, succinic and adipic) and aromatic carboxylic acids (benzoic, salicylic and cinnamic) reacted with BHBT-hydrazide under the derivatization conditions described to produce fluorescent derivatives. However, these compounds did not interfere with either the detection or the separation of the peaks for all the fatty acids , because BHBT derivatives of the compounds were eluted at 5 - 20 min under the recommended HPLC conditions. The metabolites of arachidonic acid (prostaglandin B_2, thromboxane B_2 and leukotriene B_4) also reacted with BHBT-hydrazide to yield the corresponding fluorescent derivatives and their retention times were 35 min, 15 min and 5 min, respectively, under the HPLC conditions. α-Keto acids (pyruvic,

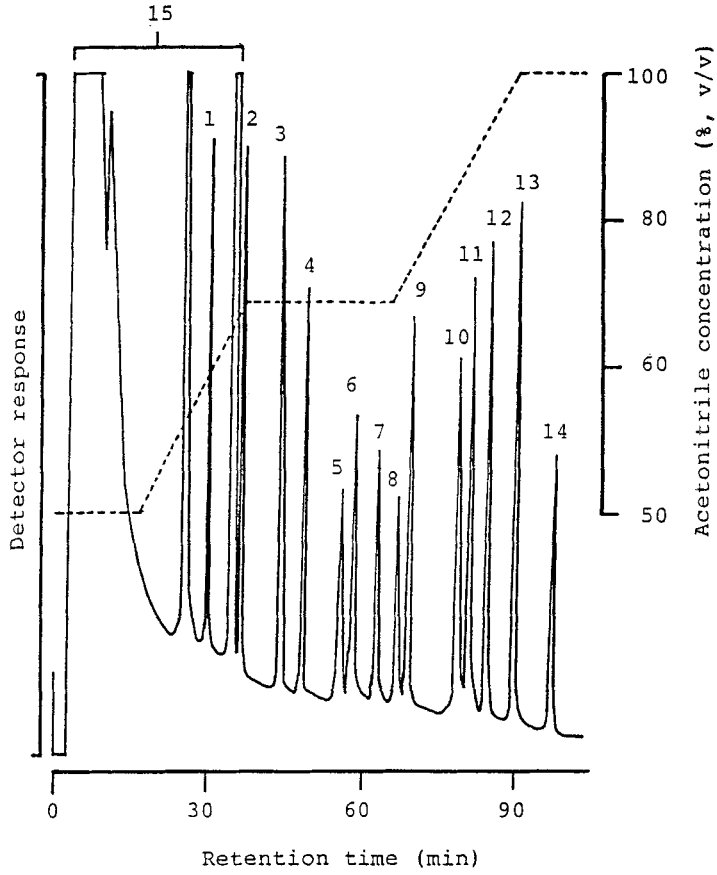


Fig. 4. Chromatogram of BHBT derivatives of fatty acids. A portion (100 µl) of a standard solution of the acids (1.0 nmol/ml each) was treated according to the described procedure. Peaks: 1=$C_{8:0}$; 2=$C_{10:0}$; 3=$C_{12:0}$; 4=$C_{14:1}$; 5=$C_{18:3}$; 6=$C_{14:0}$; 7=$C_{16:1}$; 8=$C_{22:6}$; 9=$C_{20:4}$ and $C_{18:2}$; 10=$C_{16:0}$; 11=$C_{18:1}$; 12=$C_{17:0}$; 13=$C_{18:0}$; 14=$C_{20:0}$; 15, others=reagent blank components. Mobile phase, gradient elution with aqueous acetonitrile (0-15 min, 40 %; 15-35 min, 40-70 %; 35-65 min, 70 %; 65-90 min, 70-100 %; 90-110 min, 100 %).

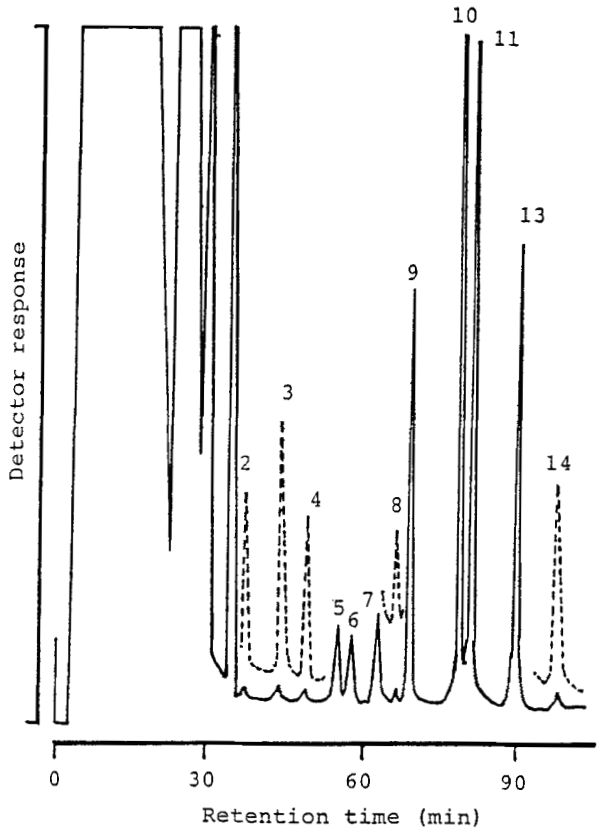


Fig. 5. Chromatogram obtained with human serum. Free fatty acid concentrations (nmol/ml serum):$C_{10:0}$=1.98; $C_{12:0}$=2.65; $C_{14:1}$=1.76; $C_{18:3}$=10.56; $C_{14:0}$=10.22; $C_{16:1}$=12.21; $C_{22:6}$=2.05; $C_{16:0}$=100.75; $C_{18:1}$=120.64; $C_{18:0}$=45.87; $C_{20:0}$=4.82.

α-ketoglutaric and phenylpyruvic) and 17 different α-amino acids did not fluoresce under the derivatization conditions employed. These observations suggested that the reagent is selective for carboxylic acids.

Application to serum free fatty acid assay

The proposed method was applied to the determination of free fatty acids in human serum.

Figure 5 shows a typical chromatogram obtained with serum. The serum levels of the acids except for $C_{8:0}$ were successfully quantified using an extremely small amount of serum (5 μl) by means of the standard addition method; $C_{8:0}$ acid was overlapped with endogenous substances in serum. The serum levels of (shown in Fig. 5) were in reasonable agreement with those by the HPLC (27,32) and GC (36) methods previously reported. The recoveries of the fatty acids (0.2 nmol per 5 μl each) added to a pooled normal serum were 95.5 - 98.3 %. This result indicates that BHBT-hydrazide is useful for the determination of carboxylic acids in biological materials.

CONCLUSION

BHBT-hydrazide is easy to synthesize and fairly stable in air and daylight, and even in the reagent solution. The reagent permits the derivatization of carboxylic acids in aqueous solution under the moderate conditions and is more sensitive (ca. 3 times) than DMEQ-hydrazide, which is one of the most sensitive fluorescence derivatization reagent. Therefore, the reagent should be useful for the detection of thermally labile carboxylic acids of biological importance at femtomole levels by HPLC.

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