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2-(2,3-Naphthalimino)ethyl trifluoromethanesulphonate as a highly reactive ultraviolet and fluorescent labelling agent for the liquid chromatographic determination of carboxylic acids

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SUMMARY

The use of 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate in the preparation of 2-(2,3-naphthalimino)ethyl ester derivatives of carboxylic acids for ultraviolet and fluorescent detection in high-performance liquid chromatography is described. The reagent is easily synthesized in two steps from 2,3-naphthalenedicarboxylic anhydride and is stable at least for 6 months at room temperature. Reactions of carboxylate potassium salts (10^{-5} *M*) with a 10-fold equivalent excess amount of the reagent proceed to completion within 10 min in acetonitrile at room temperature in the presence of 18-crown-6 as a catalyst. The derivatization procedure with this reagent has been applied succesfully to the determination of some carboxylic acids in mouse brain. The detection limits (signal-to-noise ratio = 3) with ultraviolet and fluorescent detection are 100 fmol (at 259 nm) and 4 fmol (λ_{ex} = 259 nm, λ_{em} = 394 nm), respectively.

INTRODUCTION

Carboxylic acids are widely distributed in nature and play important roles as nutritional substrates and metabolites in the living body. The carboxyl functional group is only weakly chromophoric, so carboxylates with no other adequately chromophoric structural feature must be derivatized for their sensitive detection by high-performance liquid chromatography (HPLC). Of the detection methods currently available for HPLC, fluorimetric detection is one of the most selective and sensitive. Several fluorescent labelling agents, *e.g.*, 4-bromomethyl-7-methoxycoumarin[1], 1-bromoacetylpyrene[2], 9-aminophenanthrene[3], 9-anthryldiazomethane[4] and 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone[5], have been developed for the determination of carboxylic acids by HPLC. The reactions of carboxylic

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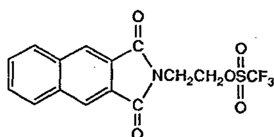
acids with these reagents are slow and/or require elevated temperatures for complete derivatization because of the poor reactivity of the carboxyl groups. Longer reaction times at elevated temperatures are unfavourable not only for simplicity in the derivatization procedures but for the determination of thermolabile substances such as α-ketocarboxylic acids.

On the other hand, the powerful alkylating abilities of perfluoromethanesulphonates are well known in the field of organic synthesis. Ingalls *et al.*[6] developed 4'-bromophenacyl trifluoromethanesulphonate as a highly reactive UV-labelling agent for carboxylic acids for HPLC determination. This reagent can derivatize the acids completely to the corresponding derivatives within 5 min in acetonitrile at room temperature in the presence of N,N-diisopropylethylamine. Recently, we have also reported the UV-labelling of carboxylic acids with 2-(phthalimino)ethyl trifluoromethanesulphonate at room temperature[7].



NE-OTf

In this work, 2-(2,3-naphthalimino)ethyl trifluoromethanesulphonate (NE-OTf) was developed as a highly reactive ultraviolet and fluorescent labelling agent for carboxylic acids in HPLC. Myristic acid was chosen as a representative carboxylic acid and the reactivity of NE-OTf towards the acid was investigated under various conditions. An attempt was made to apply the reagent to small-volume biological samples and some carboxylic acids in mouse brain were successfully determined.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a JASCO 880-PU pump, a Rheodyne Model 7125 injector valve, a JAI Model 3702 UV detector operating at 259 nm or a Hitachi F-1000 spectrofluorimeter operating at 394 nm emission and 259 nm excitation and a Shimadzu Chromatopac C-R6A integrator. Two types of analytical columns were used, a Chemcosorb 5C8 (5 μm; 150 $\times$ 4.6 mm I.D.) obtained from Chemco (Osaka, Japan) for the analysis of mouse brain samples and a Wakosil 5C18-200T (5 μm; 150 $\times$ 4.6 mm I.D.) obtained from Wako (Osaka, Japan) for all the other experiments. A Hitachi 850 spectrofluorimeter and a Hitachi 304 spectrophotometer were used for the measurements of fluorescence and ultraviolet spectra. Melting points were measured with a Yanaco melting point apparatus and were uncorrected. A Hitachi 05P-21 centrifuge and an NS-310E micro-homogenizer (Nichion Medical Instruments, Japan) were used in the preparation of mouse brain samples. An Erma ERC-3510 degasser was utilized for continuous degassing of the mobile phase.

Reagents and materials

2,3-Naphthalenedicarboxylic anhydride was obtained from Tokyo Kasei

(Tokyo, Japan). HPLC-grade methanol was purchased from Wako and used in the preparation of the mobile phases. All other chemicals were of special grade. Water was purified with a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.). Eppendorf Safe-Lock microcentrifuge tubes (2.0 ml) were used as reaction tubes. Male ddy (7–8-week-old) mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan).

Authentic 2-(2,3-naphthalimino)ethyl ester of myristic acid was prepared by reaction between N-(2-hydroxyethyl)-2,3-naphthalimide and myristoyl chloride according to the literature method[8] and identified by mass spectrometry, IR spectrophotometry and elemental analysis. This ester was used in spectrophotometric studies and in optimization studies of derivatization reaction conditions as an external reference standard.

Synthesis of NE-OTf

NE-OTf was prepared in two steps from commercially available precursors. N-(Hydroxyethyl)-2,3-naphthalimide was synthesized by modifying the literature method[9]. Thus, in a 500-ml flask fitted with a water separator and a reflux condenser were placed 9.9 g (0.05 mol) of 2,3-naphthalenedicarboxylic anhydride, 3.1 g (0.051 mol) of 2-aminoethanol and 300 ml of dry toluene. The mixture was heated for 3 h under vigorous reflux on an oil-bath. After cooling, the solid product was filtered off on a G-3 glass filter and washed with three 50-ml portions of cold water. Recrystallization from ethanol gave transparent needles of the naphthalimide: yield, 85%; m.p., 192–195°C. Analysis: calculated for $C_{14}H_{11}NO_3$, C 69.71, H 4.56, N 5.81; found, C 69.89, H 4.54, N 5.75.

To a solution of trifluoromethanesulphonic anhydride (5 g, 0.018 mol) in dichloromethane (100 ml) was carefully added dropwise a mixture of pyridine (1.4 g, 0.018 mol) and the naphthalimide (3.9 g, 0.016 mol) suspended in warm dichloromethane (100 ml) at a rate such as to keep the temperature of the reaction mixture below −5°C; the addition required about 1 h. After the addition, stirring was continued for 2 h. The resulting solution was washed three times with cold deionized water and then dried over anhydrous magnesium sulphate. After removing dichloromethane under reduced pressure, the crude product was recrystallized twice from a mixture of dichloromethane and tetrachloromethane. NE-OTf was obtained as transparent flakes: yield, 53%; m.p., 138–140°C. Analysis: calculated for $C_{15}H_{10}NSO_5F_3$, C 48.26, H 2.68, N 3.75; found, C 48.06, H 2.68, N 3.74. IR, 1200 and 1400 cm^{-1} ($-O-SO_2-$); MS, m/z = 374 (MH^+).

Derivatization procedure

A typical derivatization procedure was as follows. To 0.5 ml of a test solution of fatty acids in acetonitrile placed in a reaction tube were added 0.1 ml of 18-crown-6 (10^{-3} *M*) in acetonitrile and *ca.* 5 mg of anhydrous potassium fluoride. After vortex mixing the tube slightly, 0.1 ml of NE-OTf (10^{-3} *M*) in acetonitrile was combined with it. The mixture was vortex mixed for 10 min at room temperature. The resulting solution was allowed to stand for 30 s and an aliquot (10 μl) of the supernatant was injected directly into the chromatograph.

Preparation of mice brain samples

To the cerebrum dissected from a mouse was added margalic acid (0.1 ml) as an internal standard, and this sample was homogenized in methanol (3 ml) and centrifuged for 10 min (5000 *g*). The supernatant was removed and the residual pellet was rehomogenized in methanol. This extraction process was repeated three times. The supernatants were combined and evaporated *in vacuo*, then the residue was dissolved in acetonitrile (0.2 ml), followed by the above-mentioned derivatization procedure.

RESULTS AND DISCUSSION

It is well known that trifluoromethanesulphonate possesses an excellent alkylating ability towards nucleophilic species. However, as far as we know, there only one report has been published on the use of 4′-bromophenacyl trifluoromethanesulphonate as a UV-labelling agent for the determination of carboxylic acids by HPLC. This omission might be due to the difficulties in the synthesis and isolation of trifluoromethanesulphonate-bearing substances suitable for HPLC detection. We found that NE-OTf could be easily synthesized from commercially available materials and is stable at room temperature.

Fluorescence properties of carboxylic acid derivatives

Fig. 1 shows the excitation and fluorescence spectra of the 2-(2,3-naphthalimino)ethyl ester of myristic acid (NE-C_{14} ester) in methanol–water (9:1). The excitation and the emission maxima were at 259 and 394 nm, respectively. The effect of water concentration on the fluorescence intensity was examined. The fluorescence intensity of NE-C_{14} ester in aqueous methanol was almost constant at water concentrations of 0–30% (v/v), but decreased slightly with increasing water concentration over 30%.

Optimization of derivatization reaction conditions

Potassium carbonate is frequently used to convert free carboxylic acids into their

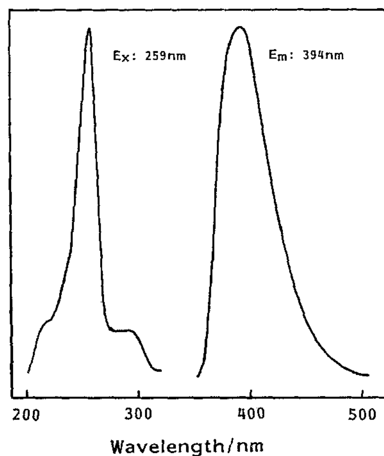


Fig. 1. Fluorescence spectra of 2-(2,3-naphthalimino)ethyl myristate in methanol–water (9:1).

carboxylate anions in derivatization systems using halogenoalkyl-type reagents. Preliminary experiments indicated that the use of potassium carbonate caused many endogenous peaks, some of which overlapped the derivative peaks of interest in sensitive detection. Shimada *et al.*[10] reported that potassium fluoride could be used in place of the carbonate for the conversion of free carboxylic acids into the carboxylate anions. We examined its use in the present derivatization system and found that the appearance of the interfering peaks was suppressed. Potassium fluoride was therefore adopted in this derivatization system.

We investigated some parameters that affect the rate of reaction and the derivatization yield, such as the reaction time and the amounts of the reagent and the catalyst. Myristic acid was chosen, owing to the suitable retention time of its derivative, as a model monocarboxylic acid in the subsequent studies. The effect of the reaction time on the derivatization yield was examined for the acid (10^{-5} *M*) both with and without 18-crown-6 as a catalyst. The reaction was completed within 10 min at room temperature with a 10-fold excess of NE-OTf in the presence of 18-crown-6. On the other hand, without 18-crown-6, it took 40 min to reach a derivatization yield of 96%. Hence a reaction time of 10 min in the presence of 18-crown-6 was adopted in the subsequent experiments.

To optimize the amounts of NE-OTf and 18-crown-6, the reactions were carried out with various equivalent ratios of each to the acid. The reaction proceeded to completion with a 5-fold excess of NE-OTf in the presence of a 10-fold excess of 18-crown-6. The effect of the relative concentrations of NE-OTf to 18-crown-6 on the derivatization yield was also examined, as shown in Fig. 2. A large excess of NE-OTf with respect to 18-crown-6 obviously brought about a decrease in the yield. On the other hand, with a ratio of 1:1, a complete derivatization yield was obtained even when a 300-fold excess of NE-OTf with respect to the acid was used in the derivatization reaction. It is therefore essential for this derivatization system to use equivalent or excess amounts of 18-crown-6 with respect to NE-OTf.

The application of the method to the determination of saturated fatty acids (C_6–C_{16}) was examined. The results indicated good linearity for the determination of

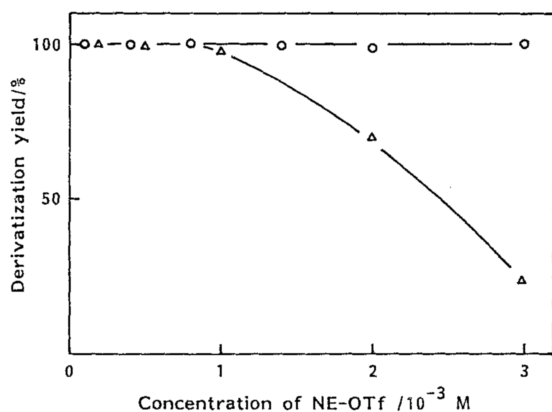


Fig. 2. Effect of concentration of NE-OTf relative to 18-crown-6 on the derivatization of myristic acid (10^{-5} *M*). ○ = 18-Crown-6 concentration = NE-OTf concentration; △ = 18-crown-6 concentration = 10^{-4} *M*.

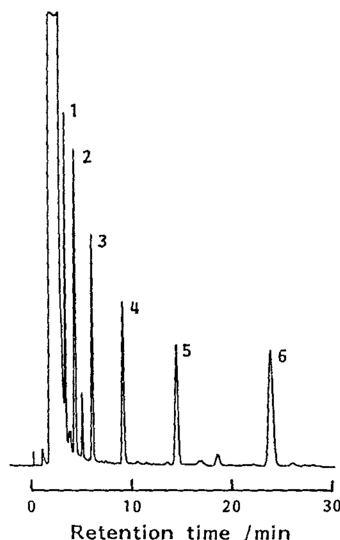


Fig. 3. Typical chromatogram of six fatty acid derivatives. Each peak corresponds to 1.0 pmol of a fatty acid. Column, Wakosil 5C18-200T; mobile phase, methanol–water (9:1); flow-rate, 1.0 ml/min; detection, fluorescence (λ_{ex} = 259 nm, λ_{em} = 394 nm). Peaks: 1 = *n*-caproic acid; 2 = *n*-caprylic acid; 3 = *n*-capric acid; 4 = lauric acid; 5 = myristic acid; 6 = palmitic acid.

each acid at seven different concentrations. The linear regression equation obtained for C_{14} acid, for instance, was $y = 0.868x + 0.471$ with a correlation coefficient $r = 0.9996$ ($2 \cdot 10^{-8}$–$1.2 \cdot 10^{-7}$ *M*), where y and x are the peak area and the concentration of the acid, respectively. A typical chromatogram of the derivatives of the acids is shown in Fig. 3.

Determination of carboxylic acid in mouse brain

As an application of this reagent to the analysis of real samples, five carboxylic acids in mouse brain were simultaneously determined according to the above procedure (Table I). The recovery of these acids in the extraction process was estimated from that of added margalic acid, which does not occur naturally in mouse brain, and

TABLE I

RESULTS OF THE DETERMINATION OF CARBOXYLIC ACIDS IN MOUSE BRAIN

Acid	*Concentration (nmol/g wet weight)*[a]	*R.S.D. (%)*[b]
Docosahexaenoic acid	18.1 ± 0.4	2.0
Arachidonic acid	79.6 ± 1.4	1.7
Palmitic acid	36.2 ± 0.7	1.9
Oleic acid	46.0 ± 1.0	2.1
Stearic acid	75.4 ± 1.6	2.1

[a] Mean ± S.D. (n = 7).
[b] Relative standard deviation (n = 7).

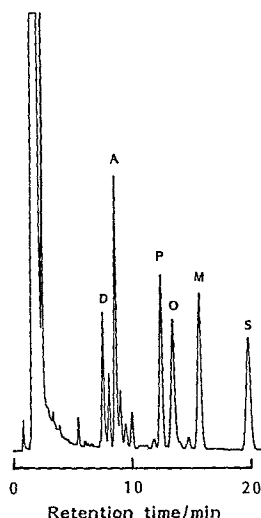


Fig. 4. Chromatogram for the determination of carboxylic acids in mouse brain. Column, Chemcosorb 5C8; mobile phase, methanol–water (87:13); flow-rate, 1.0 ml/min; detection as in Fig. 3; injection volume, 10 μl corresponding to 1/250 portions of the whole sample. Peaks: D = docosahexaenoic acid; A = arachidonic acid; P = palmitic acid; O = oleic acid; M = margaric acid (internal standard); S = stearic acid.

was consistently 95%. These derivatives were well separated from each other within 20 min on the C_8 column with methanol–water (87:13) as the eluent (Fig. 4). The values obtained for the cerebrum (0.33 g wet weight) are summarized in Table I. All the acids could be determined with relative standard deviations of *ca.* 2%.

Stability of the reagent

Solid NE-OTf was stable for at least 6 months in a light-protected desiccator at room temperature. The stability of NE-OTf in acetonitrile (10^{-2} *M*) was examined by periodically chromatographing the solution. Half of the initial peak heights were lost in 3 h at room temperature (32°C), but it was possible to keep the loss to 8% after 6 days by keeping the solution below −20°C.

In conclusion, NE-OTf can be easily synthesized from commercially available starting materials in two steps and is stable at room temperature. The labelling reaction proceeds rapidly to completion simply by mixing the reagents at room temperature, which also makes it possible to label thermolabile carboxylic acids without isomerization and decomposition. The resulting derivatives possess good chromatographic properties, strong UV absorptivity (λ_{max} = 259 nm, ε_{max} = 62 000) and intense fluorescence (λ_{ex} = 259 nm, λ_{em} = 394 nm); the detection limits (signal-to-noise ratio = 3) are 100 fmol with UV detection and 4 fmol with fluorescence detection.

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