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Chemically removable derivatization reagent for liquid chromatography: 2-(2-naphthoxy)ethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate[1]

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Abstract

A new sulfonate reagent, 2-(2-naphthoxy)ethyl 2-[1-(4-benzyl)-piperazyl]ethanesulfonate, was synthesized for analytical derivatization in liquid chromatography. The reagent consists of two main moieties, i.e., one with a fluorophore (naphthoxy) for sensitive detection after being tagged to an analyte; and the other with a tertiary amino function (a substituted piperazine) that can be removed after derivatization by acid treatment. The reagent was applied to the derivatization of caproic acid as a model analyte. The resulting derivative was analyzed by high-performance liquid chromatography with fluorometric detection. The linear range of the method for the determination of caproic acid was over 0.01–0.5 nmol, with a detection limit (signal-to-noise ratio=3) of about 0.1 pmol. The excess reagent was readily removed from organic solvent after treatment with an aqueous acid solution.

Keywords: Derivatization, LC; Naphthoxyethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate; Caproic acid

1. Introduction

Analytical derivatization coupled with various chromatographic methods [1–6] has found a wide range of applications in the fields of chemical, biochemical and other analytical sciences. Based on chemical derivatization, a detection-oriented tag, such as a chromophore or a fluorophore, can be incorporated into an analyte for trace analysis. In practice, much higher concentrations of a derivatization reagent, compared to that of the analytes, are used to derivatize the analytes at trace levels. Unfortunately, the excess reagent often interferes in the detection, especially when the derivatization reagent is also very responsive to a detector. To solve this problem, conventional approaches are applied including column clean-up [7–9], nitrogen purge [10] and adding an additional chemical [11] to remove the reagent. These treatments are usually tedious and time-consuming. Therefore, we searched for a practical method to rapidly remove excess reagent after derivatization, leading to the development of sulfonate reagents that can be removed chemically for high-performance liquid chromatography (HPLC) with UV detection, as reported previously [12,13]. In this paper, a new sulfonate reagent for HPLC with a

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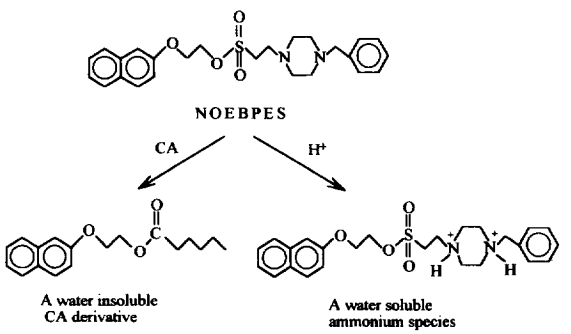

Fig. 1. Reaction scheme for NOEBPES with caproic acid (CA) and removal of excess reagent (NOEBPES). Excess reagent in the toluene was removed by aqueous acid (H^+) after derivatization.

fluorophore was synthesized, 2-(2-naphthoxy)ethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate (NOEBPE-S). Application of the reagent to the derivatization of caproic acid as a model analyte was studied. The results indicated that the derivative of caproic acid can be quantitated at the 10 pmol level by HPLC with fluorimetric detection. The excess reagent can be readily removed from organic solvent after treatment of the reacted caproic acid solution with an aqueous acid solution, as illustrated in Fig. 1. The new reagent has an auxochrome (alkoxy group) attached to the aromatic chromophore (naphthyl moiety) that usually gives favorable absorption in spectrophotometry.

2. Experimental

2.1. Materials and reagents

Caproic acid (CA), valeric acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, undecanoic acid, 9,10-dimethylanthracene, 18-crown-6 ether and N-benzylpiperazine (TCI, Tokyo, Japan), triethylamine, potassium carbonate (40–60 mesh) and silica gel 60 (70–230 mesh) (E. Merck, Darmstadt, Germany), 2-(2-naphthoxy)ethanol (Lancaster, UK), toluene, *n*-hexane, acetonitrile, chloroform and dichloromethane (Tedia, Fairfield, OH, USA), sulfuric acid and anhydrous sodium sulfate (Fisher, Fair Lawn, NJ, USA) were used without further treatment. All other chemicals were of analytical-reagent grade. Solutions of CA, NOEBPES, 18-crown-6 and 9,10-dimethylanthracene (internal standard, I.S.) were prepared by dissolving the appropriate amounts of the respective compounds in toluene.

2.2. HPLC conditions

A Waters-Millipore LC system with a U6K injector, a Model 501 pump, a Model 474 scanning fluorescence detector and a Varian 4290 integrator were used. A Nova-Pak C_{18} column (150×3.9 mm I.D.; 4 μm) and a mobile phase consisting of acetonitrile–water (60:40, v/v) at a flow-rate of 1.2 ml/min were used. The column eluate was monitored fluorimetrically ($\lambda_{ex.}$ 305 nm; $\lambda_{em.}$ 354 nm). The solvents were pretreated with a vacuum filter for degassing.

2.3. Synthesis of NOEBPES

2-(2-Naphthoxy)ethanol (9.40 g, 50.0 mmol), 2-chloroethanesulfonyl chloride (15.78 ml, 150.0 mmol) and triethylamine (27.72 ml, 200.0 mmol) were added successively to a 250-ml reaction flask containing 50 ml of dichloromethane that had been pre-cooled in an ice bath. The mixture was magnetically stirred at 0°C for 2.0 h. The reacted solution was washed successively with 10% (w/v) sodium carbonate solution (50 ml) and water (50 ml). The separated organic layer was dried with anhydrous sodium sulfate (2.5 g). The resulting residue was purified by column chromatography (27×4 cm I.D.) on silica gel 60 (ca. 200 g) with chloroform as an eluent to give 2-(2-naphthoxy)ethyl ethanesulfonate (NOEES) (5.79 g, 20.8 mmol, 42% yield) as a white powder (m.p. 82.3–83.2°C). Analysis: Calculated for $C_{14}H_{14}O_4S$, C=60.43, H=5.04, O=23.02; found, C=60.23, H=5.14, O=22.88. Electron impact mass spectrometry (MS): m/z=278 ($[M]^+$); m/z=127 and m/z=91 for naphthyl and vinylsulfonyl fragments, respectively. ^{1}H NMR (C^2HCl_3): δ 4.28–4.35 (m, 2H, ArOCH_2); 4.47–4.53 (m, 2H, CH_2OSO_2); 6.11 (d, 1H, *cis*-terminal vinyl proton, J=9.52 Hz), 6.43 (d, 1H, *trans*-terminal vinyl proton, J=16.66 Hz), 6.61 (dd, 1H, SO_2CH=CH_2, J=16.66, 9.52 Hz); 7.09–7.79 (m, 7H, aromatic H). The reaction for NOEES synthesis is based on dehydrochlorination [14] from 2-(2-naphthoxy)ethanol and 2-chloro-

ethanesulfonyl chloride using triethylamine as a catalyst.

NOEES (5.56 g, 20.0 mmol) and N-benzylpiperazine (4.32 ml, 25.0 mmol) were added to a 150-ml reaction flask containing dichloromethane (30.0 ml), which had been pre-cooled in an ice bath. The mixture was magnetically stirred at 0°C for 1.0 h. The reacted solution was concentrated in a rotary evaporator. The residue obtained was purified by column chromatography (27×4 cm I.D.) on silica gel (ca. 200 g) with ethyl acetate–*n*-hexane (1:3, v/v) as an eluent, to give NOEBPES (7.86 g, 17.3 mmol, 87% yield). A plate crystal was obtained from *n*-hexane, m.p. 74–75°C. ^{1}H NMR (C^2HCl_3): δ 2.45 (br, 8H, piperazyl H); 2.85–2.96 (m, 2H, N-CH_2-CH_2SO_2); 3.34–3.44 (m, 2H, N-CH_2-CH_2-SO_2); 3.46 (s, 2H, N-CH_2-phenyl); 4.32–4.37 (m, 2H, CH_2-naphthoxy); 4.60–4.67 (m, 2H, CH_2-CH_2-OSO_2); 7.13–7.79 (m, 12H, aromatic H). Analysis: Calculated for $C_{25}H_{30}N_2O_4S$, C=66.08, H=6.61, N=6.17, O=14.10; found, C=66.01, H=6.69, N=6.20, O=14.02. MS (fast atom bombardment): *m/z* 455 ([M+1]), *m/z* 91 (basal peak, equivalent to a tropolium ion). The reaction for NOEBPES synthesis seems to be like a Michael-type addition [15,16], with NOEES acting as a Michael acceptor.

2.4. Derivatization procedure

A 0.2-ml aliquot of a CA solution (2.5 μ*M*) was added to a 10-ml screw-capped test tube containing 0.3 ml of NOEBPES solution (3.33 m*M*), 0.1 ml of a mixed solution of 18-crown-6 (100 m*M*) and 9,10-dimethylanthracene (I.S.) (18 m*M*) and about 50 mg of potassium carbonate. The reactants were shaken at 95°C for 1.5 h. After cooling, 0.4 ml of the reacted solution was taken and washed with 1.0 ml of an aqueous solution of H_2SO_4 (1.0 *M*) by vortex-mixing for 30 s. An aliquot of the toluene layer (0.1 ml) was transferred to a test tube and purged with a gentle stream of nitrogen. The resulting residue was dissolved in 0.1 ml of acetonitrile for HPLC analysis (about 10 μl).

3. Results and discussion

The chemical removability and reactivity of NOEBPES was studied for the derivatization of CA as a test analyte. For optimization of the derivatization conditions for 0.5 nmol of CA, several parameters affecting the reaction were studied, including reaction temperature, reaction time, reaction solvent, base catalyst and the amount of NOEBPES. The parameters were evaluated by computing the peak-area ratio of the resulting CA derivative to the I.S. 9,10-Dimethylanthracene, which does not have a special functional group in its structure, was assumed to be stable and was chosen as an I.S. for the basic study; a suitable exogenous fatty acid could be tested as an I.S. for the analysis of indigenous fatty acid, if necessary.

3.1. Removability of NOEBPES after derivatization

A large excess of NOEBPES (1 mmol) was used to derivatize CA at trace levels (0.5 nmol). After derivatization, the excess reagent was simply removed by treating the reacted solution with aqueous H_2SO_4, as shown in Fig. 2. A broad and tailing peak from the excess NOEBPES appeared, with a retention time that was more than 2.0 h, when the derivatization of CA was not subjected to acid treatment. This makes the analytical time lengthy and impractical. Although in this case the reagent peak did not interfere in the analysis of CA, it interfered with the separation of other fatty acids, including decanoic acid and undecanoic acid, as shown in the selectivity test that is discussed in a later section. The problems associated with excess reagent were rapidly solved by treating the reacted solution of CA with aqueous acid.

3.2. Optimizing the parameters for derivatization

Regarding the derivatization procedure described in Section 2.4, several water-insoluble organic solvents were studied as reaction solvents at derivatization temperatures that were lower than their boiling points, to prevent the reaction system from boiling, such as toluene at 95°C, benzene at 70°C, chloroform at 50°C and dichloromethane at 30°C. The results indicated that toluene is the solvent of choice for the derivatization of CA, giving a peak-area ratio that was set as 100; the peak-area ratios obtained in

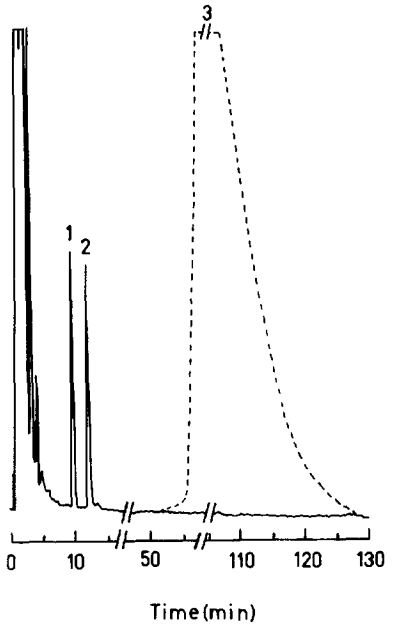

Fig. 2. Composite chromatogram of caproic acid (0.5 nmol) derivatized with NOEBPES (1 mmol), both with (solid line) and without (dashed line) acid treatment, after derivatization. Peaks: 1=the derivative of caproic acid; 2=internal standard and 3=the excess of derivatization reagent. LC conditions: Column, Nova-Pak C_{18} (15 cm×3.9 mm I.D.; 4 μm); mobile phase, 60% (v/v) acetonitrile in water; flow-rate, 1.2 ml/min; detection, $\lambda_{ex.}$=305 nm and $\lambda_{em.}$=354 nm.

benzene, chloroform and dichloromethane were 41, 4.8 and 2.6, respectively.

18-Crown-6 is essential for the derivatization of CA in the presence of potassium carbonate, since no detectable peak of the CA derivative was found in the absence of 18-crown-6. The optimal concentration of 18-crown-6 is ≥0.05 *M*, based on 0–0.2 *M* catalyst being tested. A suitable amount of potassium carbonate is ≥25 mg, based on the potassium carbonate (30–50 mesh) studied in the range of 0–200 mg. 18-Crown-6-catalyzed derivatization of carboxylic acids in the presence of potassium salt has been reported [17] for analytical and synthetic purposes.

The effects of reaction time at 70 and 95°C on the derivatization of CA are shown in Fig. 3. For derivatization at 95°C, the formation of the CA derivative reached an equilibrium in 1.5 h; whereas with a reaction at 70°C, the equilibrium was not attainable in 3.0 h and resulted in a lower yield of

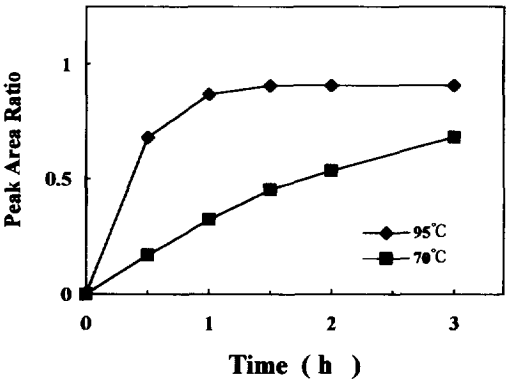

Fig. 3. Effect of reaction temperature and reaction time on the formation of caproic acid derivative.

the CA derivative, compared with the reaction at 95°C.

The molar ratio of NOEBPES to CA in the range of 500–5000 was studied on the derivatization of CA (0.5 nmol). The results indicated that a suitable molar ratio of about ≥2000 was needed for the plateau formation of the derivatization.

3.3. Stability and yield of the derivative

The stability of the CA derivative at room temperature after dissolving the derivatized residue in acetonitrile (at the end of the derivatization procedure, Section 2.4) was studied over a period of 10.0 h. No significant change in the peak-area ratio was found, indicating favorable stability of the derivative for HPLC analysis. The average derivatization yield of CA at 0.25 and 0.03 nmol was about 97% (n=3), based on the peak-area ratio of the CA derivative to the I.S., compared with that of the synthesized CA derivative to I.S.

3.4. Analytical calibration and precision

Based on the optimum derivatization conditions, the derivatization procedure for CA was formulated in Section 2.4. The quantitative applicability of the method to the determination of CA was evaluated at five different amounts of CA over the range 0.01–0.5 nmol. The calibration graph was established with the peak-area ratio of the CA derivative to I.S. as the ordinate (y) vs. the amount of CA (in nmol) as the

abscissa (x). A linear regression, $y=(0.335\pm0.004)x+(0.043\pm0.007)$ ($n=5$), was obtained with a correlation coefficient 0.999. The detection limit (at a signal-to-noise ratio of 3) of CA was about 0.1 pmol in a 0.2-ml sample (sample size, 10 μl).

The intra-day and inter-day precisions (relative standard deviation, R.S.D.) of the method were studied based on the peak-area ratios for the analysis of CA at three levels, 0.25, 0.062 and 0.031 nmol. The results in Table 1 indicated that the R.S.D.(%) ($n=7$) for the intra-day and inter-day variances were all below 2.0%.

3.5. Selectivity of the method

The selectivity of the method was briefly tested on the separation of a standard mixture of valeric acid, CA, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid and undecanoic acid, each at the 0.5 nmol level. The fatty acid mixture was derivatized according to the derivatization procedure (Section 2.4), with a larger amount of derivatization reagent (10.0 m*M*, 0.3 ml). Using these HPLC conditions, complete separation of all the fatty acids tested was obtained, as can be seen in the chromatogram shown in Fig. 4. In view of the reagent peak in Fig. 2, the more retained fatty acids, such as decanoic acid and undecanoic acid in Fig. 4, will be overlapped by the excess reagent, based on their retention times, and this can be avoided by a simple acid treatment of the fatty acid solution after derivatization.

Table 1
Precision for the analyses of caproic acid

Amount known (nmol)	Amount found[b] (nmol)	R.S.D.[c] (%)
Intra-day assay[a]		
0.250	0.252±0.002	0.79
0.062	0.062±0.001	1.61
0.031	0.031±0.0006	1.94
Inter-day assay[a]		
0.250	0.251±0.003	1.19
0.062	0.063±0.001	1.58
0.031	0.031±0.0006	1.94

[a] Intra-day data are based on seven replicate analyses and that of inter-day is from seven consecutive days.
[b] Mean±S.D. ($n=7$).
[c] Relative standard deviation.

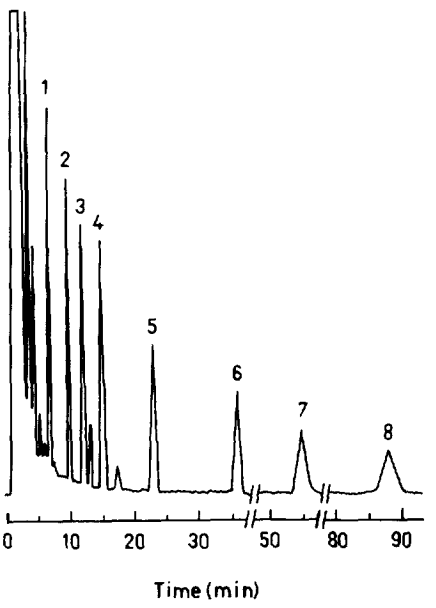

Fig. 4. LC chromatogram for a standard mixture of seven fatty acids, each at 0.5 nmol. Peaks: 1=valeric acid; 2=caproic acid; 3=I.S.; 4=heptanoic acid; 5=octanoic acid; 6=nonanoic acid; 7=decanoic acid; and 8=undecanoic acid derivatives. LC conditions were the same as in Fig. 2.

3.6. Mass spectral analysis of the derivative

The derivative of CA was synthesized by scaling up the amount of CA (0.50 mmol) with a similar procedure to that indicated in Section 2.4. The purified derivative was examined by electron impact MS (JEOL JMS-HX 110 mass spectrometer at 70 eV). The mass spectrum obtained exhibited a molecular ion at m/z 286 and a basal ion at m/z 143, equivalent to the naphthoxy fragment. This suggests that the resulting derivative is 2-(2-naphthoxy)ethyl caproate. The retention time of peak 1 in Fig. 2 is identical to that of the CA derivative synthesized.

3.7. Application

Preliminary optimization of the conditions for the extraction and derivatization of CA (1.38 nmol) spiked in human plasma was studied using similar approaches to those described in Section 3.2. The results were formulated as a protocol for the extraction and derivatization of CA as follows:

A normal plasma (200 μl) was spiked with CA in

water (27.5 μ*M*, 50 μl). After mixing, the spiked plasma was acidified (pH 0.5) with a phosphoric acid solution (2.0 *M*, 25 μl) and extracted with dichloromethane (1.0 ml). After vortex-mixing for 2 min, the sample was centrifuged at 1800 *g* for 2 min. An aliquot (0.5 ml) of the dichloromethane layer was transferred to a 10-ml screw-capped test tube containing 0.1 ml of the I.S. solution. The solution was purged with a gentle stream of nitrogen. NOEBPES solution (5.0 m*M*, 0.3 ml), 18-crown-6 solution (100 m*M*, 0.1 ml) and potassium carbonate (ca. 50 mg) were added successively to the resulting residue, which was dissolved in toluene (0.2 ml). Then the reactants were further treated as stated in Section 2.4 for the derivatization and acid treatment of CA. A typical chromatogram for the analysis of CA spiked in plasma is shown in Fig. 5c.

No significant interference from the reagent blank was found in Fig. 5a and a small peak (peak 1) in Fig. 5b from plasma blank, equivalent to the CA peak in Fig. 5c, is assigned as an endogenous CA. This indicates that the proposed method could be used to analyze fatty acids in plasma using a standard addition technique.

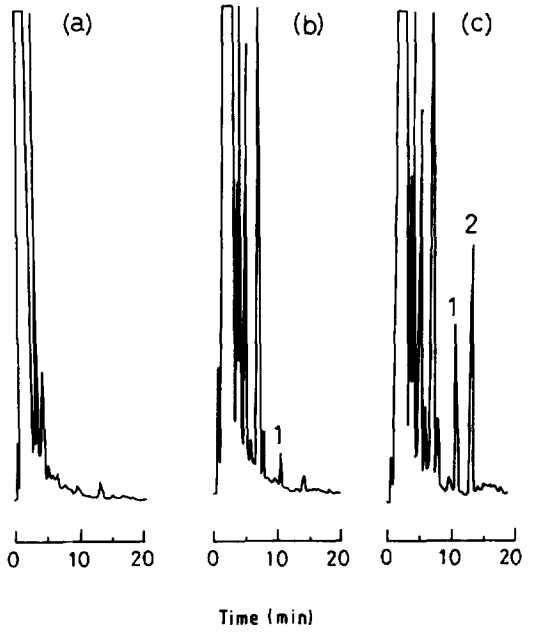

Fig. 5. LC chromatograms for (a) reagent blank, (b) plasma without spike and (c) plasma spiked with CA (1.38 nmol). Peaks: 1=derivative of CA; and 2=I.S. LC conditions were the same as in Fig. 2.

In conclusion, a new sulfonate reagent, NOEBPES, was synthesized and its application to the derivatization of CA for HPLC with fluorometric analysis was demonstrated. The results indicated that the method is selective and sensitive. The lower detection limit of detection is about 0.1 pmol; this is acceptable compared to the range of the detection limit (ca. 0.01–10 pmol) of common fluorogenic reagents [18] for carboxyl analytes. In this work, the excess reagent can be readily removed after acid treatment of CA, resulting in a simple method for analytical derivatization for liquid chromatography. Additionally, based on a preliminary TLC screening of some of the analytes tested, the reagent seems to be reactive to nucleophilic analytes with a thio group (captopril), a phenolic OH group (2,4,6-trichlorophenol) and an inorganic anion (SCN^-), in addition to a carboxyl function (fatty acid). Because new reaction spots that differed from that of the reagent blank were found for the derivatization of these analytes. Further application of the reagent to the derivatization of other analytes for sensitive detection is being studied.

Acknowledgments

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