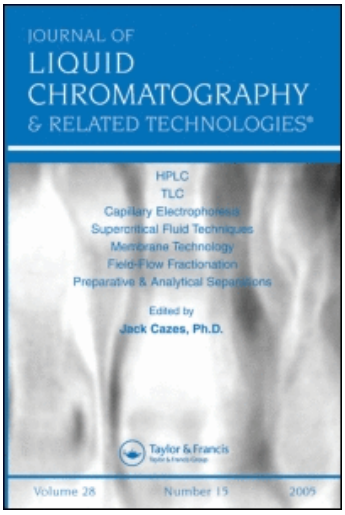

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MOZPhCSE, a New Coumarin Based Fluorescent Derivatization Reagent

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ABSTRACT

The synthesis of a new fluorescent probe 2-(3,7-dioxo-2-phenyl-3,7-dihydrochromeno[7,6-*b*][1,4]oxazin-9-yl)acetic acid succinimidyl ester was described. The reagent reacts easily with primary amines and forms highly fluorescent derivatives that can be separated and detected with an HPLC-fluorometric detector. The new reagent was evaluated using pentylamine as a model compound. The separation was achieved on a C_{18} column using a mixture of acetonitrile : ammonium acetate buffer as the mobile phase. Statistical evaluation of the methodology described reveals good linearity, precision and accuracy, and

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high sensitivity. Application of the methodology can be used for the separation of four aliphatic amines (pentylamine, hexylamine, octylamine, and decylamine).

Key Words: Coumarin; Oxazin-2-one; Derivatization; Amine; Fluorescent reagent; MOZPhCSE.

INTRODUCTION

The sensitive determination of amines is of great importance, as they are present in many biological macromolecules such as neurotransmitters, proteins, and amino acids, as well as many drugs and bioactive substances. The amino group is usually highly active so the derivatization reactions are conducted under mild conditions.

A vast number of derivatization reagents have been proposed in the literature. Among them, orthophtalaldehyde (OPA)[1–3] and fluorescamine[4–7] have found considerable applications. Especially, the second one has the advantage of giving fluorescent derivatives only with primary amines, whereas the probe is nonfluorescent itself. Both have been employed equally well to pre- and post-column derivatization reactions. Analogous to OPA, are 2,3-naphthalenedialdehyde (NDA)[8,9] and 2,3-anthracenedialdehyde (ADA).[10] Another class of fluorescent derivatization reagents are the sulfonylhalides, dansylchloride[11] being by far the most used one. Other reagents of this group are mansylchloride,[12] 2-anthracenesulfonylchloride,[13] and 1-pyrensulfonylchloride.[14] Many nitrobenzoxadiazole (NDBs)[15–17] (as the NDB-F and the NDB-Cl), as well as isothiocyanate (phenylisothiocyanate,[18] 1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate,[19] 9-isothiocyanato-acridine,[20] and isoluminol isothiocyanate[21]) derivatives have also found considerable application in amine fluorescent modification reactions. However, succinimidyl esters are considered the best class of probes for the determination of amines as they are very reactive against aliphatic amines; they form stable derivatives (amides), as well as not reacting, in general, with aromatic amines, phenols, and alcohols. Among them, are hydroxysuccinimidyl-α-naphthylacetate,[22] carbazole-9-*n*-acetyl-*n*-hydroxysuccinimide (CAHS),[23] the BIODIPY (4,4-difluoro-4-bora-3α,4α-diazaindacene),[24] fluorescein, and rhodamine succinimidyl esters.[25,26]

The coumarin nucleus has also found considerable application for synthesizing fluorescent probes.[27–29] Among these reagents, several coumarin succinimidyl ester derivatives have been synthesized as fluorescent probes, such as 2-(7-amino-4-methyl-coumarin)-acetic acid succinimidyl

ester, 7-diethylamino-coumarin-3-carboxylic acid succinimidyl ester, 7-hydroxy-coumarin-3-carboxylic acid succinimidyl ester, 2-(7-hydroxy-4-methyl-coumarin)acetic acid succinimidyl ester, 7-methoxy-coumarin-3-carboxylic acid succinimidyl ester, and 2-(7-(dimethylamino)-coumarin-4-yl) acetic acid succinimidyl ester, Alexa Fluor (7-amino-3-[2-[(2,5-dioxo-1-pyrrolidinyl) oxy]-2-oxoethyl]-4-methyl-2-*oxo*-2H-1-benzopyran-6-sulfonic acid) 350,[30] showing excellent sensitivity.

The aim of the current project was to synthesize and explore the chromatographic and fluorescent properties of a new derivatization reagent, structurally based on a combination of two chromopores, the coumarin and the benz[1,4]oxazin-2-one nucleus. Both heterocyclic nuclei exhibit intense fluorescence and they have been extensively used as fluorescent derivatization probes. For this reason, the new probe was tested using pentylamine as a model compound. The pentylamine molecule is a primary amine expected to react spontaneously with the new probe since it does not exhibit complex stereochemical hindrance as well as electronic effects.

EXPERIMENTAL

Materials and Methods

All solvents used in the analytical part were of HPLC grade and were purchased from LabScan (Ireland). The solvents used for the synthesis were of analytical grade of purity and were purchased from LabScan (Ireland). The HPLC water was doubly purified by distillation and consequent filtering and by means of a Millipore Milli-Q system. All mobile phases were degassed by filtering using a Millipore degassing device through a 0.45 μm Titan filter.

Pentylamine, hexylamine, octylamine, and decylamine were purchased from Fluka Chemie (Switzerland). All reagents used for the synthesis were purchased from Aldrich (Munchen) and were used without any further purification, except 2,4-dimethoxyaniline, which was recrystallized prior to its use.

Apparatus

A Brucker Model AC-200 instrument was used for the ^{1}H-NMR experiments. The results were processed by the Win-NMR v1 Software. A Perkin Elmer Model 883 IR instrument was used for acquiring IR spectra, the samples being treated as nujol mulls. Fluorescence spectra were acquired

on a Perkin Elmer LS-30 spectrofluorometer using spectral bandwidths of 2 nm. Sample introduction was achieved through a peristaltic pump incorporated into the spectrofluorometer. Maxima and minima were determined as averages of three consecutive measurements. UV spectra were recorded on a Perkin Elmer Lamda 7 UV spectrophotometer. Melting points were obtained by means of a Büchi melting point apparatus and are uncorrected.

The HPLC used for the chromatographic study consisted of a Spectra Physics Model SS8810 isocratic pump coupled to a Perkin Elmer LS30 spectrofluorometer equipped with a total emission accessory. Chromatograms were recorded with a Thermo Physics Model SP4270 integrator. Attenuation parameters were determined separately for every experiment. The sample was introduced via a Rheodyne injector fitted with a 20-μL loop.

Synthesis

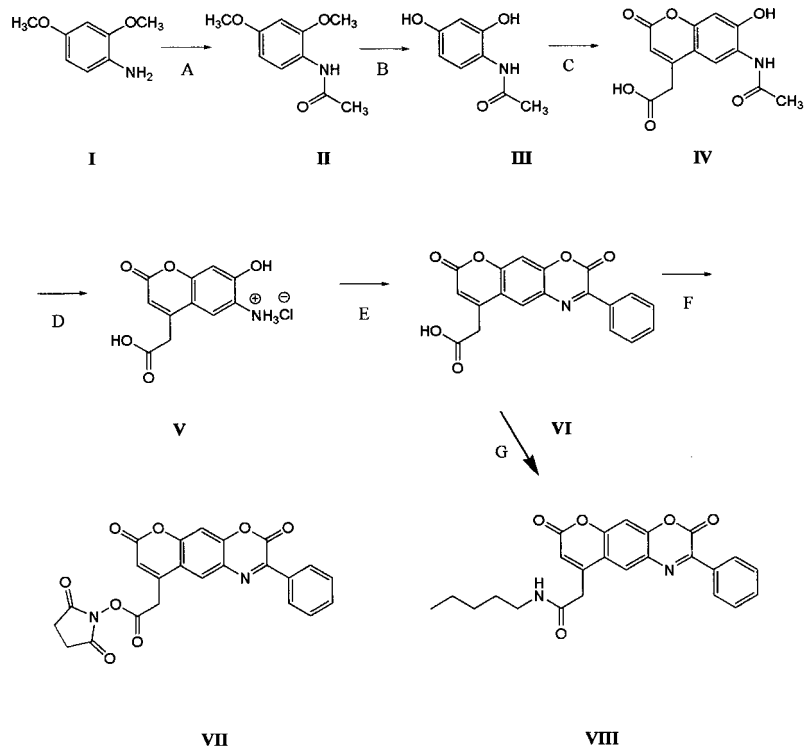
The synthesis of the synthon 2,4-hydroxyacetamide has been accomplished as described before, but for reasons of clarity has been incorporated in Sch. 1.

(6-Acetylamino-7-hydroxy-2-*oxo*-2H-chromen-4-yl)acetic Acid

Citric acid (0.08 mol) is mixed with 20 mL of H_2SO_4 for 30 min, until a uniform paste is obtained. Then, the mixture is heated slowly to 65°C, until the evolution of gas has ceased. The mixture is then brought to room temperature and is then cooled down to −15°C with an ice–salt bath. Subsequently, 0.2 mol of III is added, and the temperature is maintained low while 20 mL of sulfuric acid is added dropwise. The solution is refrigerated overnight and then poured into a mixture of ice-water. A yellow precipitate is formed, which is collected by suction filtration and recrystallized from MeOH, yielding 85%, m.p. 219–220°C. IR (cm^{-1}) (nujol): 3550–2800 (carboxylic acid and phenolic hydroxyl), 1760 (lactone), 1710 (carboxylic acid), 1675 (amide). NMR (ppm) (dmso-d_6): 2.1 (s, 3H, C*H*$_3$–CONH), 3.7 (s, 2H, –CH_2–COOH), 6.3 (s, 1H, –C*H*═CO), 6.8 (s, 1H, aromatic), 8.1 (s, 1H, aromatic), 11.0 (s, 1H, COO*H*), 11.1 (s, 1H, –CO–N*H*). Elemental analysis calculated for $C_{13}H_{11}NO_6 \cdot 1/2\ H_2O$ calc: C 54.54%, H 3.91%; found: C 54.81%, H 4.5%.

Hydrochloric Salt of (6-Amino-7-hydroxy-2-*oxo*-2H-chromen-4-yl)-acetic Acid

IV (0.0072 mol) is suspended in a solution of 40 mL methanol : 15 mL HCl and the mixture is refluxed for 30 min. The solvent was removed in



Scheme 1. Synthesis of the reagent MOZPhCSE. (A) CH_3COCl/K_2CO_3, (B) $AlCl_3$/NaCl, (C) citric acid/H_2SO_4, (D) HCl/C_2H_5OH, (E) $PheCOCOOC_2H_5/CH_3COOH$, (F) *N*-hydroxysuccinamide/DCC/DMAP, (G) $CH_3CH_2CH_2CH_2CH_2NH_2$/DCC.

vacuo and the resulting solid was washed with dioxane, yielding white solid (1.8 g, yield 82.4%) m.p. $>270^{\circ}C$. IR (cm^{-1}) (nujol): 2870–2465 (w, OH and NH_{3+} with hydrogen bond), 1705 (lactone), 1690 (carboxylic acid). NMR (ppm) (dmso-d_6): 6.3 (s, 1H, =CH–CO), 7.1 (s, 1H, aromatic), 7.6 (s, 1H, aromatic), 8.5–10.5 (2H, $-NH_2$, w), 11–12.5 (1H, –OH, w). Elemental analysis calculated for $C_{11}H_{10}ClNO_5 \cdot 1.5\ H_2O$ calc.: C 46.86%, H 3.9%; found: C 46.49%, H 3.5%.

2-(3,7-Dioxo-2-phenyl-3,7-dihydrochromeno[7,6-*b*][1,4]oxazin-9-yl)-acetic Acid

V (0.0066 mol) is suspended in 15 mL of glacial acetic acid and 2.16 mL of phenylglyoxylic acid ethylester is added. The mixture is refluxed for 90 min

and left to cool at room temperature. A green solid (1.65 g) is precipitated. Recrystallization from methanol gives a yellow solid (1.65 g, yield 64.1% m.p. 206°C). IR (cm^{-1}) (nujol): 3535–2910 (wide, carboxylic acid), 1770 (lactone), 1695 (carboxylic acid), 1650 (azomethine bond). NMR (ppm) (dmso-d_6): 4 (s, 2H, CH_2–COOH), 6.6 (s, 1H, =C*H*–CO), 7.1–7.6 (m, 4H, aromatic), 8–8.3 (m, 3H, aromatic), 115 (s, 1H, –COOH). Elemental analysis calculated for $C_{19}H_{11}NO_6$ calc.: C 65.33%, H 3.17%; found: C 65.41%, H 3.19%.

2-(3,7-Dioxo-2-phenyl-3,7-dihydrochromeno[7,6-*b*][1,4]oxazin-9-yl)-acetic Acid Succinimidyl Ester

VII (0.00055 mol) was dissolved in 25 mL of tetrahydrofuran and then cooled with the aid of an ice-bath. *N*-hydroxysuccinamide (0.069 g) dissolved in 5 mL of tetrahydrofuran is added, followed by the addition of 0.114 g of dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethyl-aminopyridine (DMAP). The mixture was sonicated for 30 min and then left overnight at 5°C. Subsequently, the cloudy solution is filtered in order to remove the precipitated dicyclohexylurea and the solvent was removed under reduced pressure, affording a white solid (0.170 g, yield 66.5%). Recrystallization from ethyl acetate gives a white solid (m.p. 252–254°C). IR (cm^{-1}) (nujol): 1750, 1730 (succinimidyl carbonyl groups), 1770, 1725 (lactone), 1685 (carbonyl group of carboxylic acid), 1650 (azomethine bond). NMR (ppm) (dmso-d_6): 2.8 (s, 4H, –CO–CH_2–CH_2–CO–), 4.2 (s, 2H, –CH_2–COO–), 6.7 (s, 1H, =C*H*–CO), 7.4–7.6 (m, 4H, aromatic), 8.1–8.2 (m, 3H, aromatic). Elemental analysis calculated for $C_{23}H_{14}N_2O_8$ calc: C 61.89%, H 3.16%; found: C 62.27%, H 3.28%.

2-(3,7-Dioxo-2-phenyl-3,7-dihydrochromeno[7,6-*b*][1,4]oxazin-9-yl)-*N*-pentylacetamide

VII (0.1 g) is dissolved in 25 mL of tetrahydrofuran, cooled with the aid of an ice bath and 0.027 g of pentylamine dissolved in 5 mL of tetrahydrofuran is added. Subsequently, 0.08 g of DCC is added and the mixture is sonicated for 30 min and then left at 5°C overnight. The formed white precipitate is filtered out and the solvent was removed under reduced pressure, affording a white solid (0.15 g, yield 62.6%). IR (cm^{-1}) (nujol): 3380 (amide), 1765 (lactone), 1730 (lactone), 1655 (amide), 1640 (azomethine bond). NMR (ppm) (dmso-d_6): 1.3 (s, 3H, –CH_3), 1.5 (m, 8H, –CH_3–), 4.3 (s, 2H, –CH_2–COO–), 6.7 (s, 1H, =C*H*–CO), 7.4–7.6 (m, 4H, aromatic), 8.1–8.3 (m, 3H, aromatic), 9.1 (s, 1H, –N*H*–).

Stock Standard Solutions

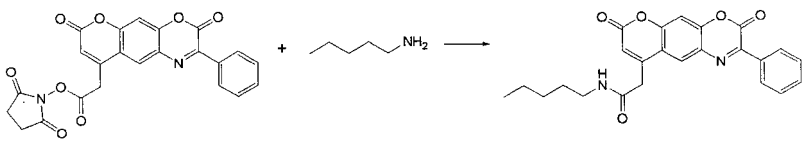
Stock standard solutions of the succinimidyl ester 460 μg/mL and pentylamine 870 μg/mL were prepared by dissolving appropriate amounts of the corresponding compounds in tetrahydrofuran. The aforementioned solutions were stored, in the dark, under refrigeration. Stock working solutions of the probe, 4.6 μg/mL and pentylamine (8.7 μg/mL), were also prepared in tetrahydrofuran. Stock standard solutions of hexylamine, octylamine, and decylamine were prepared in an analogous manner. All solutions were found to be stable for several weeks at 4°C in the dark.

Derivatization Procedure

The reaction is conducted at the 87 ng level. Thus, 80 μL of the reagent (46 μg/mL), along with 10 μL of pentylamine (8.7 μg/mL), are placed in an amber colored screw capped vial and 1 mL of tetrahydrofuran is added. The mixture is vortexed for 30 sec and left to react at room temperature for 30 min. Subsequently, 40 μL of the reaction mixture is diluted with 100 mL of mobile phase and 20 μL are injected into the HPLC. The corresponding quantity of pentylamine injected on column is 0.417 ng or 20.85 ng/mL (Sch. 2).

Chromatographic Conditions

The chromatographic separations were preformed on a reversed phase HPLC C18 column (150 × 4.6 mm, 5 μm particle diameter, Jones Chromatography, USA) using, as mobile phase, a mixture of 60 : 40 acetonitrile : ammonium acetate buffer 0.01 M. The flow rate was kept at 1 mL/min and all experiments were performed at ambient temperature. The excitation and emission wavelengths were adjusted to 376 and 448 nm and for the quantitation experiments a total emission accessory was used, using a cut-off filter at 390 nm in order to maximize sensitivity.



Scheme 2. Analytical derivatization reaction of pentylamine with MOZPhCSE.

RESULTS AND DISCUSSION

Optimization Procedure

In order to determine the optimal conditions for the derivatization reaction, the effect of critical parameters, namely the reaction temperature and time, the effect of the stoichiometry of the reaction as the effect of the solvent was evaluated. All the contributing parameters but one remained constant during this procedure, and the result was evaluated as the fluorescence signal intensity of the derivatization reaction product. The optimized value was used for the next set of optimization experiments. Every experiment was conducted in triplicate, the signal was calculated as the average value and compared vs. a blank under the same conditions.

Effect of Temperature

The effect of temperature on the derivatization reaction yield was evaluated for four temperature levels, namely 30°C, 40°C, 50°C, and 60°C and was evaluated vs. a blank sample at the same temperature. The results indicate that the rate of the product formation was maximized for the 60°C. However, higher temperature was not used as it could be near to the boiling point of the solvent, which could in turn cause repeatability problems due to condensation problems (Fig. 1a).

Effect of Stoichiometry

A stoichiometric excess of 1, 2, 4, 8, 16, and 32 : 1 was used in order to evaluate the optimal concentration of the probe. The temperature was maintained at 60°C. It seems that a stoichiometric ratio higher than 8 : 1 does not lead to increase of the reaction yield (Fig. 1b).

Effect of Time

The effect of time was assessed by analyzing reaction samples heated at 60°C at given time intervals, namely at 0, 15, 30, 45, 60, and 120 min. The reaction rate was maximized at 60 min. Heating the reaction mixture for a longer time decreases slightly the yield of the reaction, probably due to thermal degradation of the reaction product (Fig. 1c).

Effect of Solvent

Three different solvents were tested in order to optimize the fluorescent amide reaction yield, namely acetonitrile, chloroform, and tetrahydrofuran.

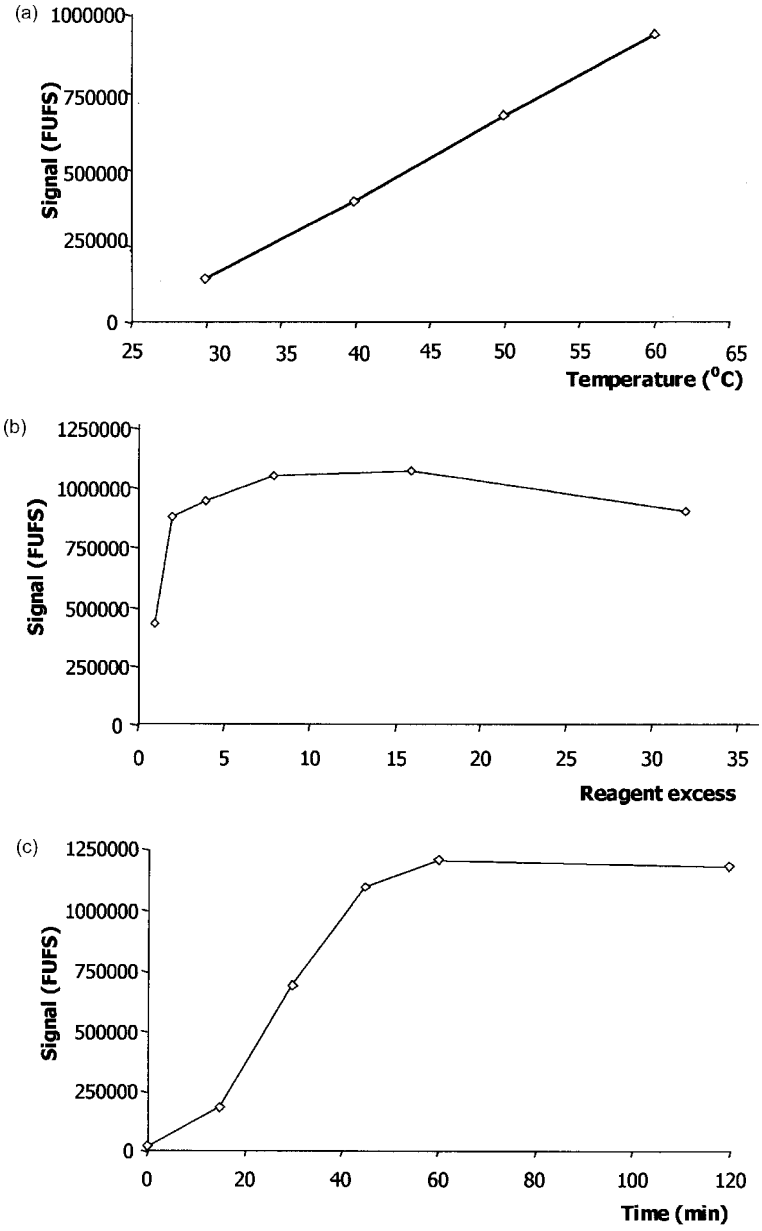


Figure 1. Effect of the derivatization reaction contributing factors on the yield of the reaction (a), time (b), stoichiometric ratio between probe and analyte (c), temperature and solvent (d).

(continued)

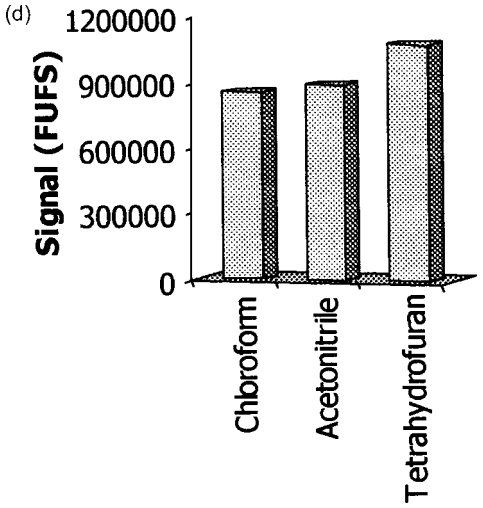


Figure 1. Continued.

The reaction rate was found to be 30% higher in the case of tetrahydrofuran, which was chosen finally as the reaction solvent.

The optimum reaction conditions selected were reaction time 60 min at temperature 60°C, using an excess of the reagent 8 : 1, and tetrahydrofuran as the reaction solvent. A corresponding chromatogram of the separation of the fluorescent amide product of the reaction from the excess of the probe is shown in Fig. 2, where the corresponding retention times are 13.09 and 14.78 min (Fig. 1d).

Design of the Probe

The coumarin (benzopyran-2-one) is well known for its fluorescent properties and it is used frequently as a fluorescent molecule, optical brightener, staining compound, or as laser dye. The incorporation of selected substituents to the molecule enhances its fluorescent properties in terms of intensity, as well as Stokes shift. The amino and the hydroxy groups are usually used for this purpose, at the six and seven positions of the aforementioned nucleus. Alkylation of these groups enhances their charge transfer properties, whereas their fusion forming an oxazine ring compensates the flexibility imposed by their free rotation. Finally, extension of the conjugated π system of the molecule, by means of addition of a benzene ring, is expected to enhance the quantum yield of fluorescence.

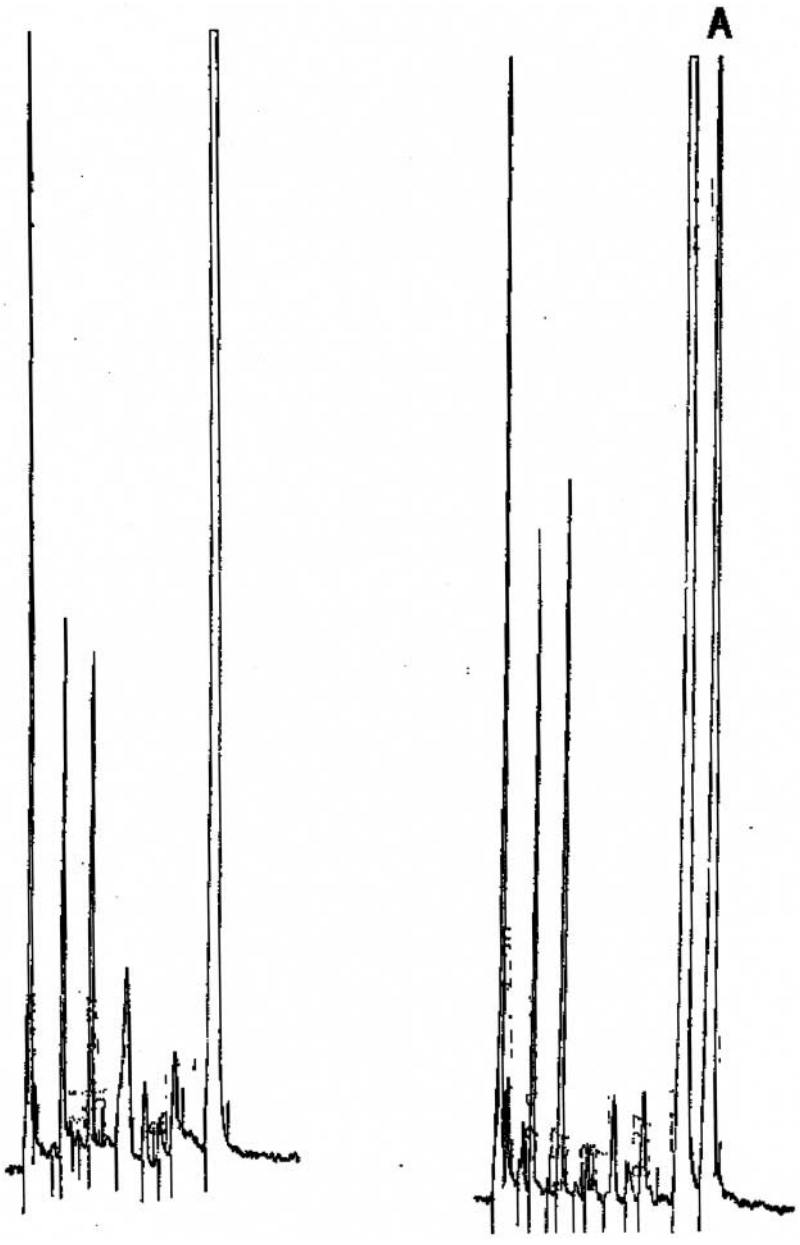


Figure 2. Chromatogram showing the separation of the pentylamine derivative from the reagent excess. The derivative (A) retention time is 14.78 min.

Statistical Analysis

In order to estimate the linearity of the proposed methodology for the determination of pentylamine, a calibration curve of concentration vs. fluorescent signal was constructed using seven calibration points covering a dynamic range from 0.5 to 32 ng/mL (0.5, 1, 2, 4, 8, 16, and 32 ng/mL). Every point of the calibration was repeated twice and the correlation coefficient was found to be 0.99992. The precision was assessed by repeating tenfold, the reaction at the 4 ng/mL level and found to exhibit relative standard deviation %RSD = 2.15, whereas the accuracy at the same level expressed as relative percentage error was found to be $E_r = 5.81\%$. The equation expressing the signal of the amide fluorescent derivative as peak area amplitude vs. concentration was:

$$\text{Signal} = 32026\ (\pm 138.6) \times C_{\text{pent}} + 4739\ (\pm 1935)$$

The detection limit of the method was found to be 10 pg (signal to noise ratio = 3) of the derivatized amine on the column, which indicates that the reagent possesses excellent sensitivity.

The methodology developed was applied to the separation of four aliphatic amines, namely pentylamine, hexylamine, octylamine, and decyl-

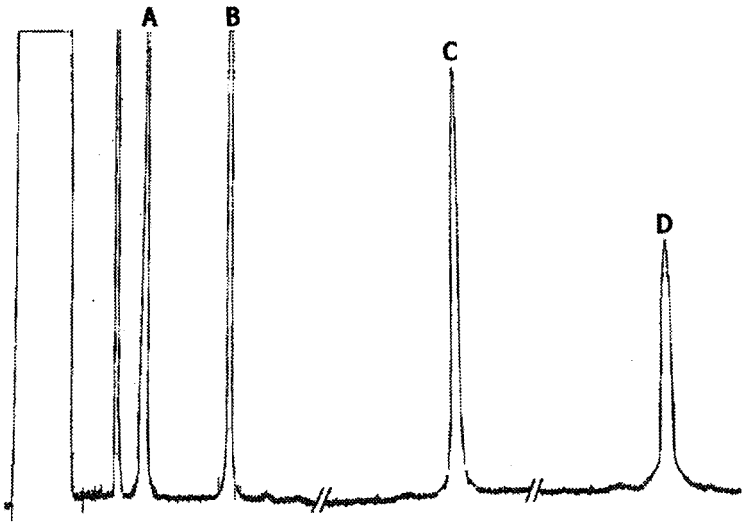


Figure 3. Chromatographic separation of four aliphatic acids (pentylamine—A, Hexylamine—B, octylamine—C, decylamine—D) as their MOZPhCSE amides. The corresponding retention times are $t_{Ra} = 21.32$ min, $t_{Rb} = 34.09$ min, $t_{Rc} = 95.14$ min, and $t_{Rd} = 210.31$ min.

amine at the 16 ng/mL level for each analyte. The log k' vs. the number of carbon atoms is linear with correlation coefficient $r = 0.999$, which reflects the increasing lipophilicity of the derivatives as the length of the acid chain increases (Fig. 3).

CONCLUSIONS

The new fused coumarin–oxazine type reagent exhibits intense fluorescence along with adequate reactivity against aliphatic amines. The chromatographic behavior of the resulting fluorescent amides enables their efficient separation. Thus, the synthesized regent can be used for the needs of trace analysis, as for example, in pharmacokinetic studies.

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