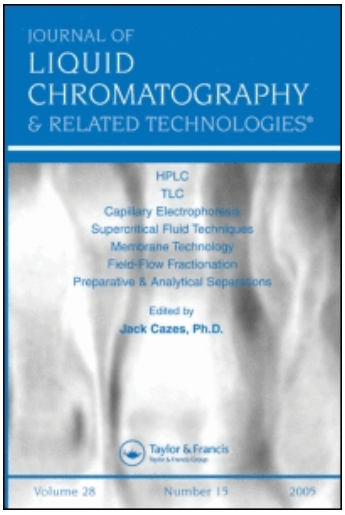

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A New Coumarin Based Fluorogenic Derivatization Reagent for Labelling Free Carboxyl Groups (Br-MOZC)

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ABSTRACT

A new coumarin based bromomethyl fluorescent probe 4-bromomethyl coumarin[a,b-c,d]-oxazine-3-one was synthesized as a fluorescent carboxylic acid derivatizing agent. The synthetic route is described, as well as its application for the determination of the model compound *n*-butyric acid. The derivatization reaction was conducted in acetone using 18-crown-6 as catalyst. The derivatization solution was separated by high performance liquid chromatography on a reversed-phase ODS C-18 column (150 × 4.6 mm i.d., 5 μm) with a mobile phase of acetonitrile and water (70 : 30, v/v) at a flow rate of 1.0 mL/min. The excitation and

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emission wavelengths were set to 435 and 345 nm, respectively. The detection limit was determined to be below 10 pg of the derivatized acid on the column. The probe has been used for the simultaneous determination of six aliphatic fatty acids.

Key Words: Derivatization reagent; NMR coumarin reagent.

INTRODUCTION

Today there is an increasing need for the determination of low levels of substances in various fields of science, such as pharmacokinetics and pharmacodynamics, environmental and residue analysis, etc. Fluorescence, which is a zero-background technique, supports this aim as it shifts the detection limits of analytical determinations more than 100–1000 times compared to UV detection,[1] exhibiting a dynamic range of more than three orders of magnitude. The increasing significance of fluorescence techniques has been demonstrated by their increasing share in chemical analyses, comparable to the corresponding share of radioanalyses.[2]

As only a small percentage (about 10%) of the known organic substances exhibit native fluorescence, the fluorescence derivatization technique (both pre- and post-column) is widely employed in analytical laboratories for the determination of a great variety of compounds exhibiting low or no fluorescence.[3] Fluorescent probes are used either in the biological field as markers and staining compounds, or in chemical analysis as fluorescence inducing reagents.

A significant number of the known organic compounds (about 8%) are found to include a carboxyl group in their structure. Among them, are many biological substances, such as prostaglandins and fatty acids, as well as numerous xenobiotics, such as antiinflamatory drugs and their metabolites. The need of their highly sensitive determination in view of meeting the needs of modern trace analysis, has forced the research in the development of new and more efficient fluorescent probes that can selectively and specifically react with such compounds.[4] The coumarin nucleus has been widely employed as such, affording a number of excellent reagents with significant commercial acceptance. Among them the bromomethyl derivatives, such as 7-methoxy-(Br-MMC),[5–7] 7-acetoxy-(Br-MAC),[8] 6,7-dimethoxy-[9] 6,7-methylendioxy-4-bromomethylcoumarin-(Br-MDC)[10,11] and 3-[(bromomethyl)phenyl]-7-(diethylamino)-2H-1-benzopyran-2-one[12] have been extensively employed for the determination of carboxylic substances like prostaglandins, NSAIDs, fatty acids, etc., as well as other types of molecules

(nucleotides etc.). These molecules have been synthesized, using the strongly fluorescent 7-hydroxycoumarin[13] as lead compound, aiming for the improvement of its fluorescent properties. The 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one molecule, which exhibits higher fluorescence than the corresponding coumarins, can be considered structurally analogous.[14] For this reason structural modifications at both the 6 and 7 positions have been made (introduction of strongly interacting with the coumarin nucleus subsituents), in order to increase the molecule's charge separation properties at the excited state, as well as to extend the delocalized molecular π electron system.[15] Recently, we have reported the synthesis and evaluation of a new fluorescent derivatization reagent, Br-MAMC, used for the determination of carboxylic acid containing substances based on the aforementioned principles.[16]

EXPERIMENTAL

Materials and Methods

All solvents used for the synthesis were of analytical grade and were purchased from LabScan (Ireland), except *N*,*N*-dimethyl formamide, which was obtained from Merck (Darmstad, Germany). The solvents used in the analytical part were all HPLC grade and were also purchased from LabScan (Ireland). The HPLC water was doubly purified by distillation and consequent filtering by means of a Millipore Milli-Q system.

Butyric, hexanoic, octanoic, decanoic, dodecanoic, and tetradecanoic acids were purchased from Fluka Chemie (Switzerland). All other reagents were obtained from Aldrich (Munich, Germany) and were used without any further purification.

The chromatographic system was comprised of a Spectra Physics Model SS8810 pump coupled to a Perkin Elmer Model LS-30 spectrofluorometer equipped with a total emission accessory, an injector fitted with 20 μL Rheodyne loop, and a Spectra Physics Model SP4270 integrator. Alternatively, a Thermo Separation spectrofluorometer was utilized for the HPLC study. The mobile phase was filtered through a Millipore degassing device with a 0.45 μm filter and degassed under vacuum prior to use.

^{1}H-NMR spectra were recorded on a Brucker Model AC-200 spectrometer (200 MHz), whereas all IR spectra were recorded on a Perkin Elmer Model 883 spectrophotometer as nujol mulls. Fluorescence measurements were performed with a Perkin Elmer LS-30 spectrofluorometer equipped with a peristaltic pump for the introduction of the sample (using spectral bandwidths of 2 nm for the acquirement of the spectra), and UV spectra were

recorded on a Perkin Elmer Lambda 7 spectrophotometer. Melting points were determined with a Büchi melting point apparatus and are uncorrected.

Of the two possible synthetic routes for obtaining the desired reagents (incorpration of the bromomethyl group during the initial formation of the coumarin nucleus or bromination of the corresponding methyl coumarin derivative[8]—the Wohl-Ziegler reaction[17]) only the first gave appreciable yield.

Briefly 2,4-dimethoxy aniline was *N*-chloroacetylated and demethylated with $AlCl_3$. Cyclization in the presence of CH_3COOK resulted in the formation of 7-hydroxybenzoxazin-3-one. Condensation of the latter with ethyl-α-bromoacetoacetate (prepared from bromination of ethylacetoacetate with molecular bromine[18] in the presence of concentrated sulfuric acid (the von Pechmann reaction) resulted in the formation of the desired probe 4-bromomethyl-coumarinobenzoxazin-3-one. Reaction of the later with butyric acid yielded the respective butylester, which was used as model compound for testing the reaction, as well as the chromatographic conditions.

2,4 Dimethoxy Chloracetanilide (II)

(Schotten-Baumann acylation), 15 gr. (0.098 mol) 2,4-dimethoxyaniline (I) were dissolved in 100 mL $CHCl_3$, and a solution of 16.7 gr. K_2CO_3 in 15 mL of water was added, forming a two-layer mixture, which was vigorously stirred. A solution of $ClCOCH_2Cl$ in $CHCl_3$ (10% excess) was added dropwise and the stirring continued for another 30 min. The two phases were separated, the organic phase washed with 10% NaOH, and the solvent removed in vacuo. The obtained brown solid was recrystallized from ethanol giving colourless needles 21.59 gr. m.p. 88–89°C (88–89°C).[19] Yield 96%.

2,4 Dihydroxychloracetanilide (III)

10 gr. (0.0435 mol) of (II), 52.2 gr. (0.391 mol) $AlCl_3$, and 20.2 gr. NaCl were thoroughly mixed and subsequently heated in an oil bath (140°C) for approximately 2 hrs until the evolution of gas had ceased. The resulting green liquid was poured in ice–water and the brownish precipitate was collected by filtration in vacuo and dried. Recrystallisation from ethyl acetate gave needles m.p. 179°C. Yield 97%. ^1H NMR (ppm)(dmso-d_6): 6,2 (dd, 1H, aromatic), 6,3 (s, 1H, aromatic), 7,2 (d, 1H, aromatic), 9,3 (2s, 2H, hydroxylic), 9,6 (s, 1H, amide).

7-Hydroxybenzoxazin-3-one (IV)

5 gr. (0.0248 mol) of (III) and 5.2 gr. (0.055 mol) of CH_3COOK in 125 mL water were boiled, under reflux, for 3 hrs and the reaction mixture was allowed to cool at room temperature. The precipitate was collected by suction filtration and recrystallized from absolute ethanol (white needles 3.5 gr, 85.36%) m.p. 208°C [208°C (19)]. Yield 85.36%.

4-Bromomethyl coumarin[a,b-c,d]-Oxazine-3-one (V)

2 gr. (0.012 mol) of (IV) and 2.54 gr. (0.012 mol) of ethyl α-bromoacetoacetate were mixed until a uniform paste was obtained. Concentrated sulphuric acid (50 mL) was added dropwise while the mixture was kept in an ice–salt bath (−10°C). After the addition of the acid, the mixture remained at 5°C overnight. Subsequently, the reaction mixture was poured in a water–ice mixture, so that the temperature was kept low and the pale yellow precipitate was collected by filtration. The filtrate was washed with cold water until washings became neutral (3.3 gr). Recrystallistion from DMF/methanol twice gave a yellow solid m.p. >270°C Yield 88%. ^{1}H NMR (ppm)(dmso-d_6) 4,6 ppm (s, 2H,), 4,7 ppm (s, 2H, $-CH_2$), 6,6 ppm (s, 1H, $-CO-CH=C-$), 7,1 (s, 1H, aromatic), 7,3 ppm (s, 1H, aromatic), 11 ppm (s, 1H, amide) IR (nujol mull) 3400 (amide stretch), 1730 (lactone), 1680 (lactam). Elemental analysis calculated for $C_{12}H_8BrNO_4$ C 46.63% H 2.60% found C 47.01% H 2.64%.

Butyryl Ester of V (VI)

Butyric acid (0.280 mL 0.0032 mol), V (0.25 gr. (0.0008 mol.), and 1.5 gr. dry K_2CO_3 were refluxed in anhydrous acetone for 3 hrs. The solvent was removed in vacuo and 100 mL of cold water was added, giving a yellow solid collected by suction filtration (0.2 gr). Recrystallisation from ethyl acetate gave yellow prisms m.p. >270°C. Yield 78%. ^{1}H NMR (ppm)(dmso-d_6): 0.9 ppm (t, 3H, $-CH_3$), 1.3 ppm (m, 2H, $-CH_2-$), 1.6 ppm (t, 2H, $-CH_2-$), 4.7 ppm (s, 1H, $-CH_2-$), 5.2 ppm (s, 1H, $-CH_2-$), 6.4 ppm (s, 1H, $-CO-CH=C-$),), 7.1 (s, 1H, aromatic), 7.2 (s, 1H, aromatic), 11 ppm (s, 1H, amide). IR (cm^{-1})(nujol): 3345 (amide), 3415 (amide), 1742 (lactone), 1660 (lactam) elemental analysis calculated for $C_{12}H_8BrNO_4$ C 60.57% H 4.76% found C 60.92% H 4.75%

The synthetic route is represented briefly in Fig. 1.

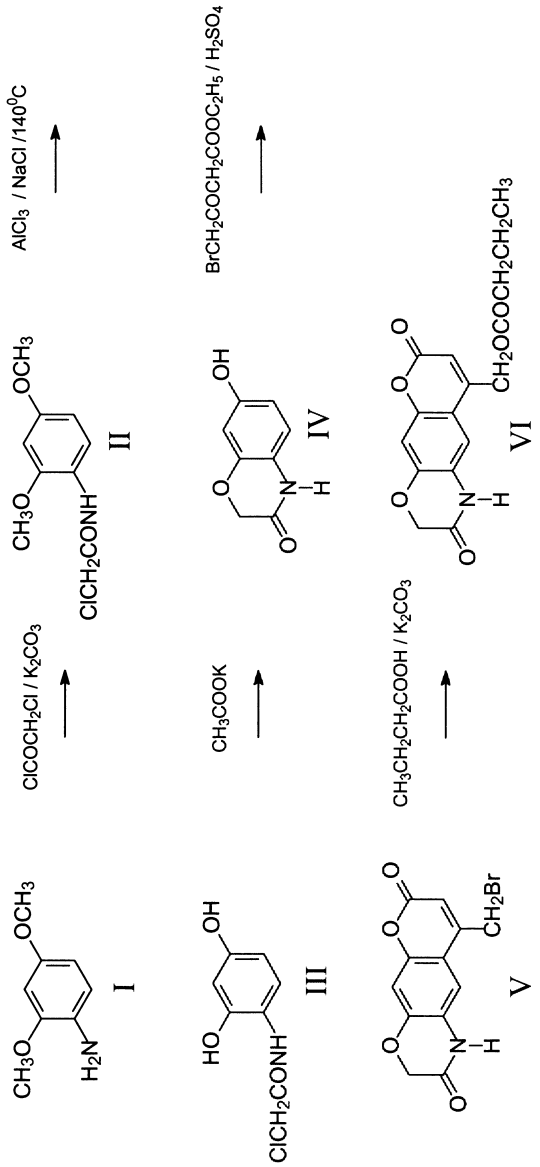

Figure 1. Synthesis of 4-bromomethyl coumarin[a,b–c,d]-oxazine-3-one (Br-MOZC) (V) and its butyryl ester (VI).

Initial Analytical Derivatization Procedure

The chromatographic separation experiments were performed using the following derivatization procedure: 100 μL of the appropriate acid (4.4 mg/mL), 80 μL of reagent (V) (3.1 mg/mL), 2 mg finely powdered K_2CO_3 were placed in 4-mL screw-capped vial along with 30 μL of 18-crown-6 ether (3 mg/mL) (used as a phase transfer catalyst) and the volume was filled up to 1 mL with acetone. After vortexing for 60 sec, the reaction mixture was heated for 20 min at 50°C. A 20 μL aliquot was drawn, diluted to 5 mL of mobile phase, and injected. The corresponding concentration was 16 ng/mL of butyric acid.

The rest of the experiments were performed using a 40 μg/mL solution for Br-MOZC and appropriate volumes of boutyric (model compound) or the other five aliphatic acids (1.0 μg/mL) used in this study, as described below. An appropriate volume (25–500 μL) of the boutyric acid solution reacted with 50 μL of the Br-MOZC solution (40.0 μg/mL) and corresponding aliquots of acetone were added to reach a volume of 1000 μL. The reaction occurred in the presence of 15 μL of 1.0 μg/mL 18-crown-6 solution and 2 mg of a fine suspension of K_2CO_3, into an amber-colored micro-reaction vessel (3.0 mL). The reaction mixture was allowed to stand for 30 min at 25°C. Consequently, 100 μL of this reaction mixture were drawn and diluted with 100 μL of the mobile phase; a 20-μL aliquot was injected into the HPLC. The same procedure was repeated for the derivatization reaction of all five fatty acids.

The reaction mixture was chromatographed on a C18 column (ODS 150 × 4.6 mm, 5 μm ID) using acetonitrile–water (70–30, v/v) as the mobile phase. The flow rate was 1 mL/min and all chromatographic experiments were conducted at ambient temperature. Excitation and emission wavelengths were adjusted to 345 and 435 nm respectively. Chromatograms were recorded using fluorescent attenuation estimated in every experiment. A corresponding chromatogram is shown in Fig. 2.

RESULTS AND DISCUSSION

The unsubstituted coumarin nucleus is fluorescent only in acidic values of pH when the carbonyl oxygen of the lactone is protonated and therefore its $n \rightarrow \pi^*$ excitation is unfavorable.[20] The fluorescent properties of the molecule are demonstrated intensely when an electron donor substituent is introduced at the 6 or 7 position.[21] The most successful examples of fluorescent coumarins in the literature include molecules of this type. A further enhancement of the fluorescent properties is observed when two

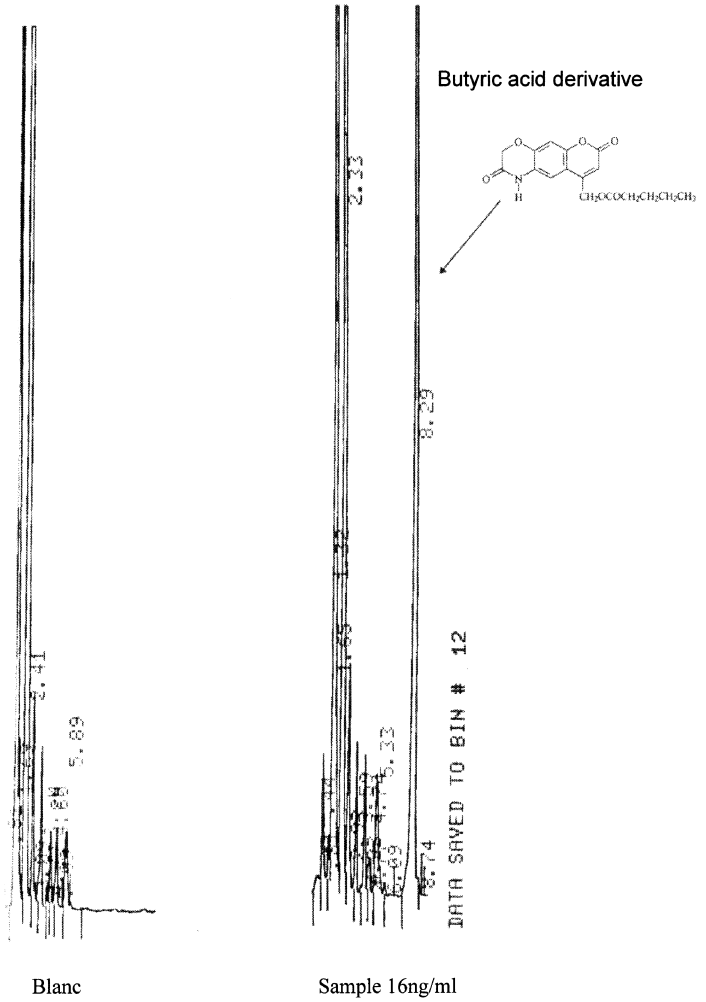

Figure 2. Chromatographic separation of the butyryl ester of Br-MOZC eluted at $t_R = 8.29$ min reversed-phase HPLC on an ODS C-18 column; eluent acetonitrile–water (70–30, v/v); flow rate 1 mL/min; $\lambda_{ex} = 345$ nm and $\lambda_{em} = 435$ nm.

electron donors are present in both the 6 and 7 positions. It was due to this fact that Farinotti et al.[9] introduced two methoxy groups in the aforementioned positions, increasing, thus, the charge transfer properties of the molecule (Fig. 3.I). In order to increase the rigidity of this structure, thus enhancing the

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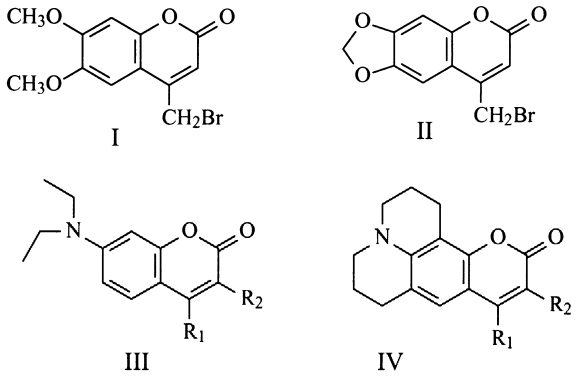

Figure 3. Coumarins used as fluorescent probes in analytical derivatisation reactions. Molecules II and IV represent improvements of molecules a and c as their substituents are immobilized by their fusion to a dioxolane (II) or two piperidine (IV) nuclei.

fluorescence by increasing the quantum yield, Naganuma and Kawahara[11] have synthesised the corresponding methylendioxy analogue (Fig. 3.II). The rotation ability of the two methoxy groups is restricted through stereochemical hindrance, so that low loss of excitation energy is observed through vibrational relaxation.[22]

An analogous approach was followed for the synthesis of luminarins[23–25] (Fig. 3.IV) (analogues of the 7-dialkylamino coumarins (Fig. 3.III)), where the two alkyl substituents of the amine group were incorporated into two piperidin nuclei, promoting the rigidity of the structure and minimising any energy losses due to vibrational relaxation.

These two features, i.e., two electron donors and rigid planar structure were incorporated to the Br-MOZC molecule. Two different interacting groups were introduced: an etheric oxygen analogous to the methoxy group at position 7 and an amide at position 6. The introduction of an amide group instead of a disubstituted amine (a feature common to many fluorogenic-laser used labels as in the luminarins) was chosen with the prospect of extension of the conjugation between the former and the benzene ring, leading to the delocalisation electron pair of nitorgen. The fusion of both the ether function and the amide in an oxazinone ring contributed to the increase of inflexibility of the structure and the expansion of the resonance structures, contributing, thus, to the extension of the delocalised π bond system of the molecule as determined by semi-empirical and *ab initio* molecular modeling studies.[26]

Optimisation of the Reaction Conditions

The reaction conditions were optimised for a number of major contributing factors, namely temperature and time, absence and amount of the catalyst used (18-crown-6), stoicheiometric ratio of the reagent to the analyte, etc. Butyric acid was used as a model molecule, as it doesn't exhibit any significant stereochemical hindrances or complex electronic effects, so that the effectiveness of the derivatization reaction can be adequately estimated. Additionally, it doesn't possess any native fluorescence, which could interfere with the evaluation of the sensitivity of Br-MOZC as a fluorophore. In every step of the optimisation process, all the parameters affecting the reaction yield (e.g., time, temperature etc.) but one were kept constant. Every point was injected in duplicate vs. a blanc at the same conditions. The optimisation of the derivatisation reaction was performed to the higher concentration of the calibration curve.

Temperature Dependence

The derivatization was conducted at four different temperatures [Fig. 4(a)]. The reaction rate is maximal at 40°C but it slightly decreases above that limit, presumably due to thermal decomposition of the derivative.

Kinetics

The reaction was monitored for 120 min with 5 min ramps for the first 20 min. After 60 min the reaction yield was maximized and and remained stable to further heating, indicating that this is the optimum reaction time for the derivatization reaction [Fig. 4(b)].

The effect of 18-crown-6, which has been used as a phase transfer catalyst, has been evaluated in the kinetics of the reaction. The derivatization was monitored in the presence and absence of the catalyst in an effort to simplify the reaction system. The results [Fig. 4(d)] indicate that the reaction rate is significantly lower when the crown ether is not present, but it doesn't exhibit considerable effect on the yield of the reaction.

The stoichiometric relation between the reagent and the analyte was determined by titration of a standard amount of the latter with increasing amounts of Br-MOZC under the reaction conditions described. It can be

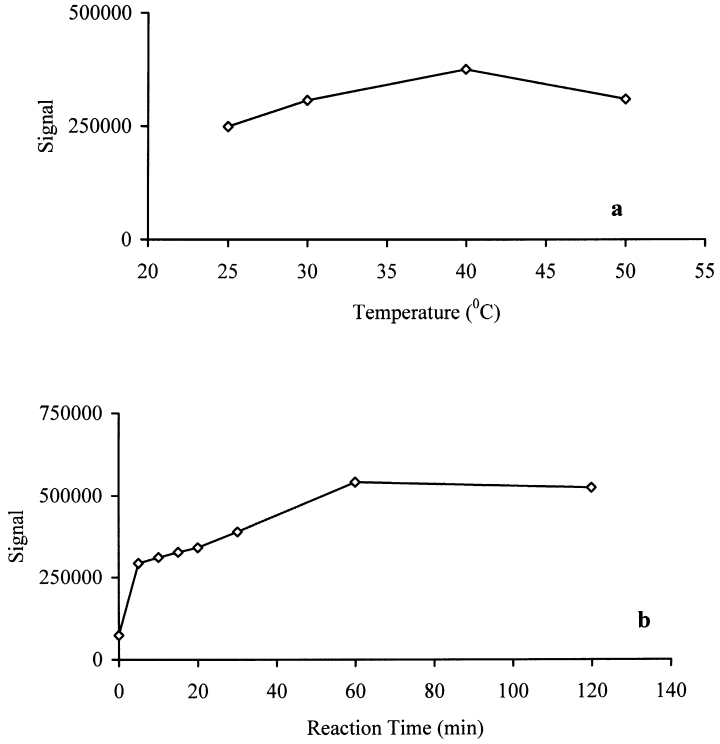

Figure 4. Influence of (a) temperature, (b) reaction time, (c) stoicheiometric ratio, (d) 18-crown-6 of Br-MOZC-boutyric acid on the derivatization of boutyric acid with Br-MOZC. In every step of the optimisation procedure all the contributing factors but one remain constant and the optimised value is used to the next experiment.

(continued)

deduced that the reaction forms a plateau for an excess of 30 : 1 ratio of reagent to analyte [Fig. 4(c)].

The role of the solvent nature was also investigated. The reaction was carried out in acetonitrile (the second more favorable solvent for this reaction), as well as in chloroform under the conditions established. The yield, which was amplified in acetone, showed a small decrease in acetonitrile, whereas in chloroform was minimal (less than 20% of acetone yield). This supports the suggestion that the reaction happens through S_{N2} mechanism where a polar aprotic solvent facilitates the dissociation of the active complex.

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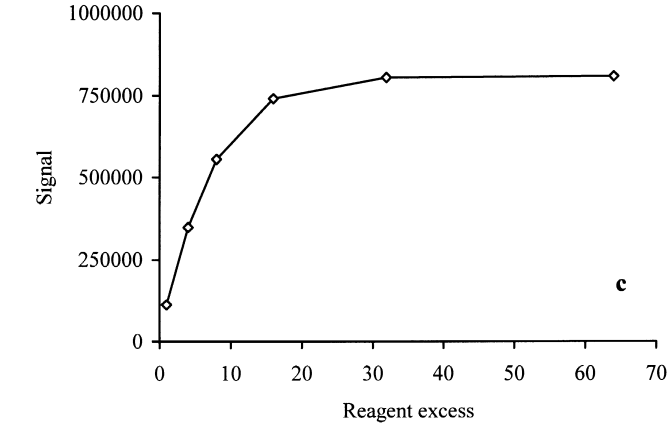

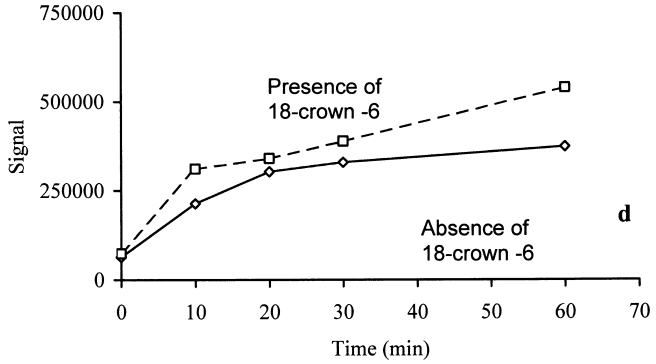

Figure 4. Continued.

Statistical Evaluation

The calibration curve of the boutyric acid concentration vs. the fluorescence signal (expressed as the peak area) was linear within the 3.6 ng/mL to 225 pg/mL range exhibiting a correlation coefficient of 0.9994 (calibration equation $S = 64784(\pm 335) \times C_{\text{but}} + 3510(\pm 623)$).

The accuracy of the analytical determination, based on the calculation of the coefficient of variation, was determined to be 2.58% for the 450 pg/mL reaction after 10 repetitions.

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The yield of the analytical reaction was established, comparing the peak areas obtained from injecting a sample of the analytical reaction at the 1.8 ng/mL level and a sample made from dilutions of the synthetic ester at the same level, and was found to be 94.8%.

The method's limit of detection was determined to be below 10 pg of the derivatized boutyric acid on column (assuming the S/N ratio = 3). The derivatization reaction for the assay for the LOD was repeated three times.

Separation of Six Aliphatic Acids

The method was applied to the simultaneous determination of six aliphatic acids, each having an ethylene unit difference from the previous one (namely butyric, hexanoic, octanoic, decanoic, dodecanoic, and tetradecanoic acid). A new mobile phase (60/40 acetonitrile–water, v/v) was utilized; not giving baseline resolution for the first peak but the separation can be conducted in reasonable time (Fig. 5).

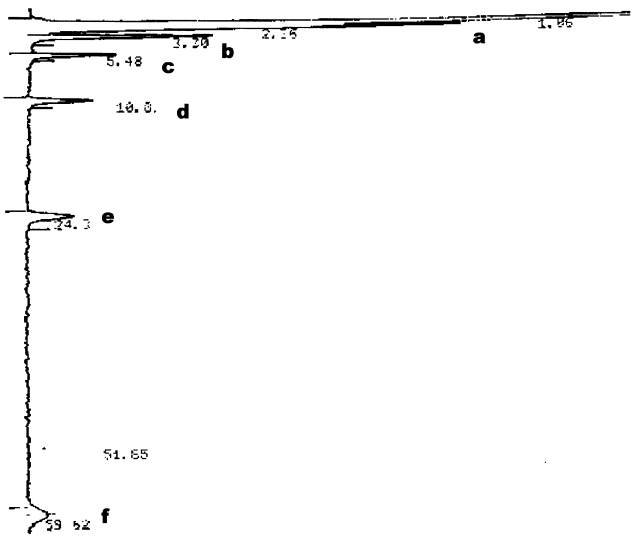

Figure 5. Chromatographic separation of 6 aliphatic acids (butyric—a, hexanoic—b, octanoic—c, decanoic—d, dodecanoic—e and tetradecanoic—f) as their corresponding Br-MOZC esters. The corresponding retention times are $t_{Ra} = 2.36$ min, $t_{Rb} = 3.30$ min, $t_{Rc} = 5.48$ min, $t_{Rd} = 10.01$ min, $t_{Re} = 24.3$ min, and $t_{Rf} = 59.62$ min.

Table 1. Relationship between the number of carbon atoms and $\log K'$. Their relationship is linear described by the equation $\log k' = 0.2035x - 1.3609$ (x = number of carbon atoms) with $r^2 = 0.9998$.

Relationship between carbon atoms and retention data			
Number of C atoms	Retention time	Capacity factor k'	$\log k'$
4	2.36	0.268817	−0.57054
6	3.3	0.774194	−0.11115
8	5.48	1.946237	0.289196
10	10.01	4.38172	0.641645
12	24.3	12.06452	1.08151
14	59.62	31.05376	1.492114

Note: $t_0 = 1.86$ min.

Table 1 represents the retention time and the capacity factors of the derivatives. The relation between the number of the carbon atoms of each acid and $\log k'$ was found to be linear ($r = 0.9998$) reflecting the increase of lipophilicity and molecular surface for each derivative.

CONCLUSION

Br-MOZC was synthesised as a part of an ongoing program of our department, aiming at the synthesis and evaluation of fluorophore molecules as derivatization reagents in HPLC processes. It was planned with the aid of both semi-empirical and *ab initio* quantum mechanical calculations[26] and was tested with a model compound (butyric acid) showing remarkable fluorescent properties. Furthermore, Br-MOZC was employed in initial experiments for the determination of NSAIDS like acetylsalicylic acid and enalapril for pharmacokinetic studies with promising prospects.

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