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Visible Diode Laser-Induced Fluorescence Detection in Liquid Chromatography after Precolumn Derivatization of Amines

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To fully exploit the attractive visible diode laser-induced fluorescence (LIF) detection technique in column liquid chromatography (LC), covalent labeling procedures need to be developed which are compatible with near-infrared fluorescence. For this purpose, several red-absorbing labels containing a single succinimidyl ester functionality were synthesized and used for the derivatization of primary and secondary amines. Oxazines/thiazines, squaraines, and dicarbocyanines were examined as red-absorbing fluorophores. The quality of the LC–diode LIF system for the analytes concerned is illustrated by the concentration detection limit of the labeled *n*-octylamine (a test compound), which was as low as 2×10^{-12} M. As expected in view of the limited reactivity of the succinimidyl ester group, the analyte concentrations required to obtain quantitative reaction with the label were equal to or higher than 2×10^{-8} M. To show the applicability of the method to biological samples, urine was spiked with 5×10^{-7} M 1-adamantanamine, extracted, derivatized with a dicarbocyanine-based label, and analyzed by LC–diode LIF.

There are several reasons to develop near-infrared (near-IR) fluorescence detection schemes in column liquid chromatography (LC). The cheap, stable, and small-sized visible diode lasers can be utilized; even in complicated matrices like urine or plasma, very few fluorescent interferences are present; Raman scatter intensity is reduced 50-fold by using excitation at, e.g., 670 nm instead of 250 nm. Unfortunately, absorption in the red region is possible only for highly conjugated compounds, which are difficult to synthesize and are often (photo)labile.

Southwick et al.[1] described the synthesis of a number of red- and near-IR-absorbing covalent labeling reagents but did not study the derivatization reaction at low analyte concentrations. In our group, the applicability of a similar label for trace analysis was recently demonstrated: 2-mercaptobenzothiazole was determined at concentrations below 1.0×10^{-8} M using a dicarbocyanine-based reagent with an iodoacetamide reactive group.[2] We have now attempted to synthesize and apply red-absorbing covalent labeling reagents with another functionality, i.e., a succinimidyl ester, for trace analysis. Succinimidyl esters are well-known for their acylating properties with amino groups at slightly alkaline pH (cf. ref 3); their reactivity is relatively low, so the achievable analyte detection limits will be somewhat less favorable, a situation comparable with that observed previously for the detection of thiols.[2]

Emphasis is on three types of fluorophores which have received much attention in the recent literature, i.e., oxazines/thiazines, squaraines, and dicarbocyanines (Figure 1). To utilize them as a basis for labeling agents, the following aspects are important: (i) the possibility to attach a single carboxylic acid to the fluorophore skeleton; (ii) the ease of conversion to a succinimidyl ester; (iii) the (high) fluorescence quantum yield in partially aqueous solvent mixtures (as used in reversed-phased LC); and (iv) the rate of the derivatization reaction with primary and secondary amines.

Oxazines and thiazines (extinction coefficients $\epsilon = (0.2–1.0) \times 10^5$ L mol^{-1} cm^{-1}) such as Nile Blue and Azure B have been used as covalent and noncovalent labels for proteins.[4] Their absorption maxima shift to longer wavelengths with an increasing number of alkyl or aryl substituents on the two amine functionalities.[5] A problem is the decrease in fluorescence quantum yield for the alkyl- or phenyl-substituted compounds, especially in aqueous solutions.[6–8] Reaction of the primary amine group with carboxylic acids has the same effect.[9] Hence, commercial oxazines and thiazines are less useful as labeling agents. Only if a primary alkyl spacer is introduced between the amine and a second functional group, which is a laborious procedure, can such a dramatic decrease of the fluorescence quantum yield be circumvented.[10]

In one study, an Azure B-based label provided with a succinimidyl ester reactive group was synthesized and used for the fluorescent labeling of amines (Figure 1, label).[4] Unfortunately, no spectroscopic data were reported, and excess analyte was used during derivatization.

Squaraines ($\epsilon = (1.0–2.0) \times 10^5$ L mol^{-1} cm^{-1}) are 1,3-disubstituted squaric acid derivatives, resulting from the condensation of squaric acid (3,4-dihydroxy-3-cyclobutene-1,2-dione) and

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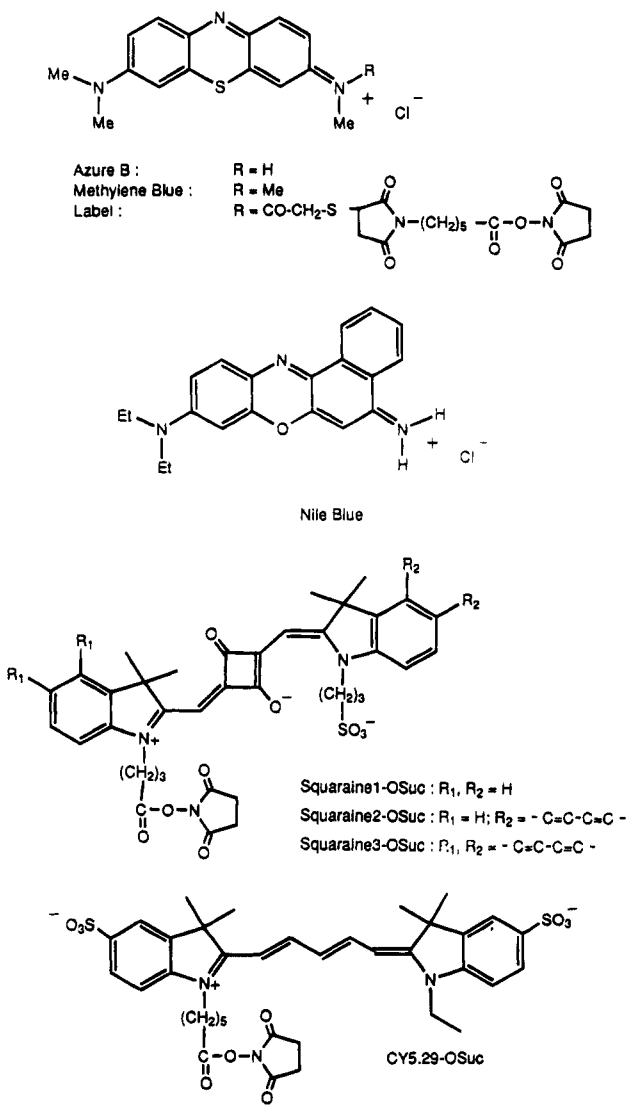

Figure 1. Examples of red-absorbing oxazine/thiazine, squaraine, and dicarbocyanine dyes.

active compounds such as pyrroles, indoles, or anilines.[11] Squaraines derived from indoles (Figure 1, squaraine1-OSuc) show strong absorption in the red region of the spectrum and allow the attachment of a single active group.[12] They are highly soluble in water and show a reasonable fluorescence quantum yield in aqueous mixtures. Unfortunately, much of the relevant literature has been reported in patents.[13]

Dicarbocyanines ($\epsilon = (1.5-2.5) \times 10^5$ L mol^{-1} cm^{-1}) (Figure 1 CY5.29-OSuc, numbering according to ref 14) have been the subjects of many studies, but the introduction of reactive groups to make them suitable for covalent labeling has been carried out only recently.[1] Mujumdar et al.[14] have published a study on sulfoindocyanine succinimidyl esters, which were developed to enhance water solubility compared with earlier reported succinimidyl esters.

The test compounds in this study are both primary and secondary *n*-alkylamines. To show the applicability of LC–diode laser-induced fluorescence (LIF) to biological samples, 1-adamantanamine was determined in spiked urine. 1-Adamantanamine is an antiviral agent that is active against various strains of influenza virus and is increasingly used as an anti-Parkinson agent.[15] After extraction from a biological fluid, it is usually determined by GC with electron-capture detection[16] or by derivatization with a fluorescent label and analysis by LC.[17,18] The detection limits in biological samples are $(0.5-1) \times 10^{-7}$ M for both methods.

EXPERIMENTAL SECTION

Instrumentation. Isocratic LC of the labels and the derivatized amines was performed with mixtures of acetonitrile–water containing 0.1% trichloroacetic acid. The eluent was delivered at a rate of 0.75 mL min^{-1} by a Model 300 high-precision pump (Gynkotek, Germering-München, Germany). A Valco injection valve, equipped with a 25 μL loop, was used to inject the samples on a 250 × 3.1 mm i.d. C-18 (5 μm particles) Vydac (Hersperia, CA) analytical column. For detection, diode LIF was applied,[19] using a 3 mW, 635 nm diode laser (Philips CQL840/D, Eindhoven, The Netherlands) in combination with a laser-line selector (Applied Photophysics, London, England), except for the labels that contain at least one benz[*e*]indole group. For these compounds a 10 mW, 670 nm diode laser (Toshiba TOLD 9215(S), Tokyo, Japan) was used without the laser-line selector.

Absorbance measurements were performed on a DU-64 spectrophotometer (Beckman, Anaheim, CA). Fluorescence spectra were recorded on a LS-50 spectrofluorometer (Perkin-Elmer Nederland, Gouda, The Netherlands) using red-sensitive R928 photomultipliers. Spectra were automatically corrected for differences in excitation efficiency in the red region of the spectrum. Extinction coefficients were determined from absorbance values of weighed samples of dried material. Fluorescence quantum yields (Φ_f) were obtained using the red-absorbing dye DOTCI (Radiant Dyes, Wermelskirchen, Germany) as standard ($\Phi_f = 0.33$ in methanol).[20]

Reagents. HPLC-grade methanol and acetonitrile were purchased from J. T. Baker (Deventer, The Netherlands), and 1,3,3-trimethoxypropene and 1,1,2-trimethyl-(1*H*)-benz[*e*]indole were purchased from Kodak (Rochester, NY). The amines, including 1-adamantanamine hydrochloride, were obtained from Janssen Chimica (Tilburg, The Netherlands) and distilled before use. All other organic chemicals were obtained in the highest available grade from Aldrich Chemical Co. (Newark, NJ).

A raw sample of squaraine-1-OH, obtained from Dr. T. L. Tarnowski (Syntex, Palo Alto, CA), was converted into squaraine-1-OSuc and used to study the possibility of labeling with water-soluble squaraines.[21]

Synthesis of the Derivatization Labels. *Oxazines.* Since red-absorbing thiazines have a low fluorescence quantum yield in aqueous solutions (<0.05 for Azure B and Methylene Blue in

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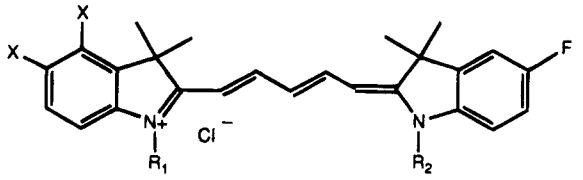
Table 1. Structural Elements of Various Dicarbocyanine Labels



compound	X,X[a]	functionality (F)	R_1	R_2
CY5.11-OSuc	H,H	$CH_2COOSuc$	$(CH_2)_4SO_3^-$	C_2H_5
CY5.11a-OSuc	−C=C−C=C−	$CH_2COOSuc$	$(CH_2)_4SO_3^-$	C_2H_5
CY5.11b-OSuc	H,H	COOSuc	$(CH_2)_4SO_3^-$	C_2H_5
CY5.11c-OSuc	−C=C−C=C−	COOSuc	$(CH_2)_4SO_3^-$	C_2H_5
CY5.13-OSuc	H,H	$CH_2COOSuc$	$(CH_2)_4SO_3^-$	$(CH_2)_4SO_3^-$
CY5.13a-OSuc	−C=C−C=C−	$CH_2COOSuc$	$(CH_2)_4SO_3^-$	$(CH_2)_4SO_3^-$
CY5.13b-OSuc	H,H	COOSuc	$(CH_2)_4SO_3^-$	$(CH_2)_4SO_3^-$
CY5.13c-OSuc	−C=C−C=C−	COOSuc	$(CH_2)_4SO_3^-$	$(CH_2)_4SO_3^-$

[a] H,H and −C=C−C=C− designate an indole and a benz[*e*]indole substituent, respectively.

water−methanol (50/50 v/v)), attention was focused on oxazines. As was stated in the introduction section, strict requirements apply to the substituents of the oxazine skeleton because of their influence on the fluorescence quantum yield. As a result, though several procedures are known for the synthesis of oxazines,[10,22,23] no suitable labeling compound could be made without forming large amounts of side products.

Squaraines. Three squaraines containing carboxylic acid functionalities were considered.[13] The carboxylic acid precursor of squaraine-1-OSuc was synthesized, utilizing information provided by Tarnowksi et al.,[21] and purified. Squaraine-2-OSuc and squaraine-3-OSuc form interesting alternatives because they can be excited at longer wavelengths. The synthesis of squaraine-2-OSuc was performed similar to that of squaraine-1-OSuc, using 1,1,2-trimethyl-(1*H*)-benz[*e*]indole instead of 2,3,3-trimethyl-(3*H*)-indole for the nonfunctionalized side of the molecule. Squaraine-3-OSuc was obtained by replacing both indole functionalities by benz[*e*]indole groups. For all three squaraines, quantitative conversion of the carboxylic acids to the corresponding succinimidyl esters was achieved by reaction with dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF).

Dicarbocyanines. CY5.29-OSuc was synthesized according to Mujumdar et al.,[14] using malonaldehyde bis(phenylimine) dihydrochloride; however, many red-absorbing side products were observed (total, ~18%). Therefore, an alternative approach was chosen, using 1,3,3-trimethoxypropene for the synthesis of the corresponding carboxylic acid (CY5.29-OH), followed by reaction with disuccinimidyl carbonate (DSC) in DMF (see below, Synthesis of CY5.11a-OSuc). The amount of side products so obtained was negligible (<0.3%); less than 10% of unreacted carboxylic acid was present.

Various other dicarbocyanine labels were synthesized. Their general structure and functionalities are given in Table 1. The precursors of the succinimidyl esters are carboxylic acids, denoted as CY5.*xx*-OH. To obtain CY5.11-OH and CY5.13-OH, the procedure of Southwick et al.[1] was followed. To achieve a red shift in the absorption maxima, CY5.11a-OH and CY5.13a-OH were synthesized using the approach outlined previously.[2] Compounds with the carboxyl group directly connected to the aromatic ring were obtained by replacing (2,3,3-trimethyl-(3*H*)-indol-5-yl)-acetic acid[24] with 2,3,3-trimethyl-5-carboxy-(3*H*)-indole;[25] these are denoted CY5.11b-OH, CY5.11c-OH, CY5.13b-OH, and CY5.13c-OH.

The spectroscopic properties of the dicarbocyanine labels and the squaraine-based labels are presented in Table 2. The extinction coefficients are fairly high, and the fluorescence quantum yields (in the aqueous solution considered) are only 30% lower than in pure acetonitrile. The absorption maxima are in line with those reported by Southwick et al.[1] for similar compounds. The fluorescence quantum yield is somewhat lower for the compounds in which the carboxylic acids is directly linked to the aromatic ring.

Succinimidyl esters can readily be obtained for all dicarbocyanines in Table 2 by reaction with DSC in DMF or, alternatively, DCC in acetonitrile. As a typical example, the synthesis and purification of CY5.11a-OSuc is detailed below; the various steps are depicted in Figure 2.

Synthesis of CY5.11a-OSuc (II). To obtain **II**, 250 mg (0.72 mmol) of **I** (from ref 2) was dissolved in 1.8 mL of acetic acid, and 845 mg (6.4 mmol) of 1,3,3-trimethoxypropene was added under stirring. After 20 min, 9 mL of diethyl ether was added, which resulted in the separation of a very viscous brown liquid. The mixture was stirred for another 15 min and cooled in an ice bath. The bright yellow supernatant was decanted, and the brown residue was dissolved in a small amount of acetic acid−methanol (50/50) and kept under nitrogen to prevent the formation of (blue) symmetrical dicarbocyanine with two sulfonate groups. If no acetic acid is added, a green color appears. Cold diethyl ether was added until the solution became hazy. Next, the solution was cooled in ice water until a yellow precipitate was formed. This precipitate was collected by filtration over a paper filter. The yield of **II** was 74%.

Subsequently, 221 mg (0.535 mmol) of **II** was dissolved in 11 mL of methanol, after which 200 mg (0.535 mmol) of **III** and 129 mg (2.2 equiv) of potassium acetate were added; this resulted in the immediate appearance of a strong blue color. After overnight

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Table 2. Spectroscopic Properties of Various Squaraine- and Dicarbocyanine-Based Labels in Acetonitrile–Water (50/50)

compound	$\lambda_{ex\ max}$[a] (nm)	$\lambda_{em\ max}$[a] (nm)	Φ_f[b]	ϵ_{max}[b] (L mol^{-1} cm^{-1})
squaraines				
squaraine-1-OSuc	633	643	0.12	205 000
squaraine-2-OSuc	652	664	0.14	225 000
squaraine-3-OSuc	671	686	0.10	235 000
dicarbocyanines[c]				
CY5.29-OSuc	649 (649)	668 (667)	0.28 (0.27)	220 000 (218 000)
CY5.11-OSuc	650 (650)	672 (671)	0.14 (0.15)	215 000 (215 000)
CY5.11a-OSuc	664	686	0.21	185 000
CY5.11b-OSuc	651	671	0.11	210 000
CY5.11c-OSuc	670	686	0.19	180 000
CY5.13-OSuc	650 (651)	670 (672)	0.16 (0.16)	200 000 (205 000)
CY5.13a-OSuc	668	684	0.22	185 000
CY5.13b-OSuc	658	678	0.12	195 000
CY5.13c-OSuc	670	689	0.18	180 000

[a] Accurate within 1 nm. [b] Accurate within 5%. [c] Numbers in parentheses are for the corresponding *n*-octylamine derivatives.

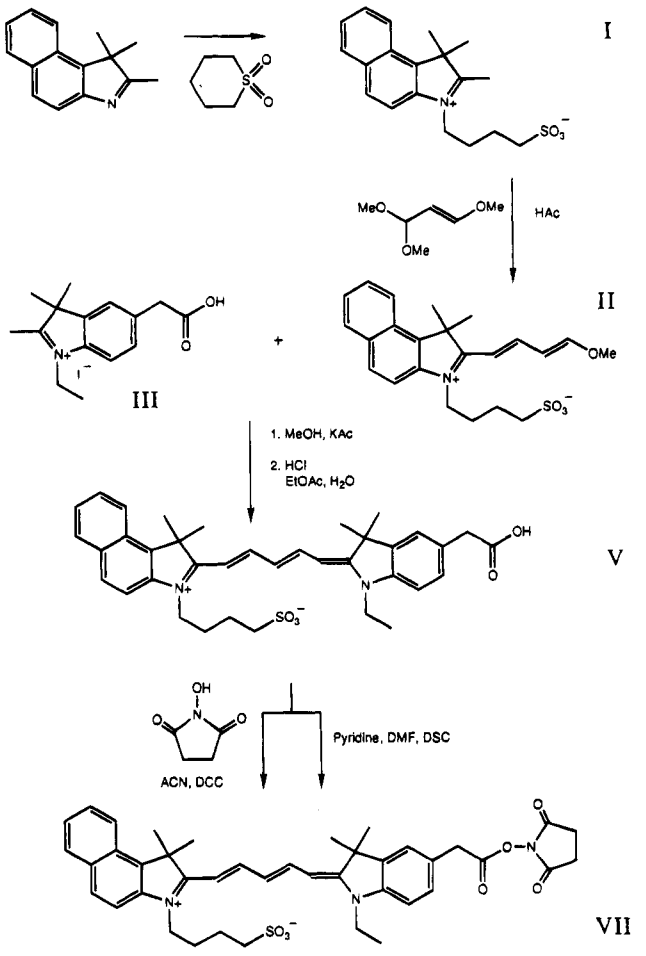

Figure 2. Procedure for the synthesis of a typical dicarbocyanine label. For details, see text.

stirring of the mixture at room temperature, 1 equiv of 1 M hydrochloric acid was added. The reaction mixture was stirred for 15 min, and the solvent was removed by evaporation under reduced pressure. The solid was washed three times with 5 mL of ethyl acetate; 3 mL of demineralized water was added, and the suspension was stirred for 6 h to remove both the (small amount of) symmetrical dicarbocyanine (**IV**) and the potassium chloride formed. After the supernatant was decanted, the remaining water was evaporated under reduced pressure, and the finely distributed solid was dried over P_2O_5. Thin-layer chromatography, under the conditions given in Table 3, showed that next to CY5.11a-OH (**V**), the corresponding methyl ester (**VI**) was formed as a side product. The total yield of **V** was 60–80%.

Table 3. R_f Values on Silica and C-18-Bonded Silica for the Various Blue Compounds Formed during the Synthesis of CY5.11a-OSuc[a]

compound	C-18	silica
symmetrical dye with two SO_3^- (**IV**)[b]	0.82	0.30
CY5.11a-OH (**V**)	0.72	0.36
CY5.11a-OMe (**VI**)	0.53	0.94
CY5.11a-OSuc (**VII**)	0.60	0.84

[a] Eluent with C-18-bonded silica was acetone–water (67/33). Eluent with silica was chloroform–methanol (70/30). [b] Formed as a side product from compound **I** and 1,3,3-trimethoxypropene.

Compound **VI** was removed by preparative LC over a silica (85 g, 70–100 mesh, Merck) column by gradient elution with chloroform–methanol, starting with 90/10 and increasing the methanol concentration during the run. Under these conditions, **VI** eluted first, followed by **IV** and **V**. Compounds **V** and **VI** were collected separately. **VI** can be readily converted into compound **V**: 242 mg (0.386 mmol) of methyl ester **VI** was dissolved in 1 mL of demineralized water, and 1 equiv (15 mg) of sodium hydroxide was added. The mixture was heated to 55 °C for 30 min. After the mixture cooled to room temperature, 2 equiv of hydrochloric acid was added, followed by a small amount of acetonitrile; this resulted in the precipitation of carboxylic acid **V**. Finally, the precipitate was collected on a paper filter and dried over P_2O_5.

To obtain CY5.11a-OSuc (**VII**), 30.7 mg (0.050 mmol) of **V**, 1.5 equiv (8.39 mg) of *N*-hydroxysuccinimide, and 1.5 equiv (10 mg) of DCC were dissolved in 1 mL of acetonitrile. After being stirred overnight, the reaction mixture was filtered over a paper filter to remove dicyclohexylurea. Acetonitrile was evaporated at room temperature. After trituration with 5 mL of ethyl acetate, the residue was dissolved in a minimal amount of acetonitrile and reprecipitated by the addition of diethyl ether. LC with fluorescence detection (for conditions, see Table 4) showed a conversion exceeding 90%. Further purification of **VII** was performed by elution with chloroform–methanol (90/10) over a column containing 5 g of silica (70–200 mesh). The total yield of solid **VII** was 60–80%. The identity of the product was checked by NMR and

Table 4. Retention Times (min) of Some Primary and Secondary Amine Derivatives of Three Red-Adsorbing Succinimidyl Esters[a,b]

	CY5.11a-OSuc	squaraine-1-OSuc	CY5.29-OSuc
carboxylic acid	3.9	3.0	3.3
succinimidyl ester	4.4	4.2	4.0
label-NHC_4H_9			3.9
label-$N(C_2H_5)_2$			4.1
label-NHC_5H_{11}	5.5	5.3	5.5
label-NHC_6H_{13}	6.1	6.0	8.2
label-$N(C_3H_7)_2$	6.3	6.2	8.4
label-NHC_7H_{15}	6.8	7.2	13
label-NHC_8H_{16}	7.3	9.0	20
label-$N(C_4H_9)_2$	7.3	9.1	20
label-NHC_9H_{19}	11	13	31
label-$NHC_{10}H_{21}$	15	21	44

[a] The LC system described in the Experimental Section was used to purify the labels and to separate them from the derivatized analytes. Specific eluents: for CY5.11a-OSuc, acetonitrile–water (70/30) + 0.1% trichloroacetic acid; for squaraine-1-OSuc and CY6.29.OSuc, water–acetonitrile (70/30) + 0.1% trifluoroacetic acid. [b] All analytes are primary or secondary alkylamines.

mass spectrometry after ion exchange as described by Southwick et al.[1]

RESULTS AND DISCUSSION

Considering the three types of near-IR fluorophores, it is obvious that thiazines and oxazines provide limited perspective for the development of appropriate labels. Therefore, attention will be focused on the squaraines and the dicarbocyanines mentioned in Table 2. Since succinimidyl esters can undergo hydrolysis, the stability of the labels in aqueous solutions has to be studied, and next, the rates of hydrolysis and of amine labeling have to be compared. Acetonitrile was selected as the organic solvent because rapid ester formation occurs in methanol. Depending on their solubility, stock solutions of the labels (1 mg/100 μL) were made in either acetonitrile or DMF and stored at 4 °C.

Hydrolysis rates were measured at room temperature (20 °C) after dilution of the stock solution of the label to 5×10^{-5} M in acetonitrile–50 mM phosphate buffer (50/50) in the pH range 7.0–9.5. The amounts of succinimidyl ester and carboxylic acid were determined by LC with fluorescence detection. Since the absorption/fluorescence characteristics of the compounds concerned are quite similar, direct quantitation of the relative amounts was done on the basis of the peak area of the recorded peaks. For pH values greater than or equal to 9.0, less than 10% active ester is still present after 3 min. At pH 8.5, 30% of the original ester concentration remains after the same period of time, while at pH values less than or equal to 8.0, the label is relatively stable. These characteristics are representative for all the labels considered.

Evidently, the amine labeling rates have to be studied under conditions similar to those described above. Immediately prior to labeling, stock solutions of the succinimidyl esters were diluted to 5×10^{-5} M in acetonitrile–water (50/50); 250 μL of this solution was added to 250 μL of 1×10^{-6} M *n*-octylamine in acetonitrile–50 mM phosphate buffer (50/50). Aliquots of 25 μL were analyzed by LC with fluorescence detection after dilution with acetonitrile–100 mM phosphate buffer (pH 4.0) to 5×10^{-10} M. The highest total yield was observed at pH 8.5, which was 10% higher than that at pH 9.0 and 9.5. For all pH values greater than 8.0, the reaction yield reached a plateau within 5 min, mainly due to the high hydrolysis rate of the label. Although the label is relatively stable at pH 8.0, a minimum of 15 min was required to obtain a total yield similar to that obtained at pH 8.5. Obviously, even longer reaction times were required at lower pH values. All labels showed similar reactivity at room temperature; at higher temperatures the reaction rates were higher, but little improvement was observed in the total yield.

The conclusion is that, in water, low concentrations of analyte cannot be derivatized quantitatively because of the rapid hydrolysis of the succinimidyl ester, an inherent disadvantage of this functional group. We therefore performed derivatizations in pure acetonitrile or, if solubility problems were encountered, in acetonitrile–DMF mixtures. Since extensive dilution with eluent was applied before LC analysis, the influence of DMF on the LC separation was negligible.

With CY5.11a-OSuc as test compound, the formation of side products under water-free conditions was studied by LC with fluorescence detection; triethylamine was used as the activating base. No hydrolysis was observed under the conditions examined. Distillation of triethylamine, acetonitrile, and DMF was necessary to prevent the formation of unidentified side products.

Derivatization in acetonitrile was studied between 20 and 70 °C using 0.01–2% triethylamine. Before LC was applied, the reaction mixture was diluted with acetonitrile–phosphate buffer (pH 9.0). This resulted in rapid hydrolysis of the succinimidyl ester, and the carboxylic acid thus formed elutes earlier, so the chromatographic window for the derivatized analyte is significantly increased.

To establish the analytical performance of the derivatization technique, 1×10^{-6} M *n*-octylamine was derivatized with 2.5×10^{-5} M CY5.11a-OSuc in acetonitrile. Optimum reaction conditions were derived from reaction surfaces (28 data points each), using reaction time (seven values) and base concentration (four values) as the two *X*-variables and the relative signal as the *Y*-value, obtained at four temperatures (20, 40, 60, and 70 °C): reaction time, 45 min; triethylamine concentration, 0.5%; and reaction temperature, 60 °C. Samples were injected after dilution of the derivatized compound to a concentration of 5×10^{-10} M in acetonitrile–50 mM phosphate buffer (50/50) (pH 8.5). The equation for the calibration curve over the concentration range of 2×10^{-8} to 1×10^{-5} M was

$$Y = 33.2\ (\pm 10.3) + (1.11 \times 10^9)X\ (\pm 0.010)$$
$$(r^2 = 0.997) \qquad (7\ \text{data points},\ n = 3)$$

where *X* and *Y* are the concentration and the relative peak height, respectively. The injection and reaction repeatability were determined at a concentration of 1×10^{-10} M derivative after labeling 5×10^{-7} M *n*-octylamine (n = 6). The relative standard deviations were 1.3% and 3.5%, respectively, which is quite satisfactory.

In the concentration range of 1×10^{-5}–2×10^{-8} M, the derivatization yield was over 90%, the calculation based on a comparison of the peak areas of the derivative and a standard solution of the label. However, below 1.5×10^{-8} M, the yield started to decrease rapidly. The detection limit for the *n*-octylamine derivative was 2.0×10^{-12} M (S/N = 3; N, peak-to-peak noise) under the LC conditions of Table 4 if solutions were prepared by diluting a solution of the amine derivatized at a

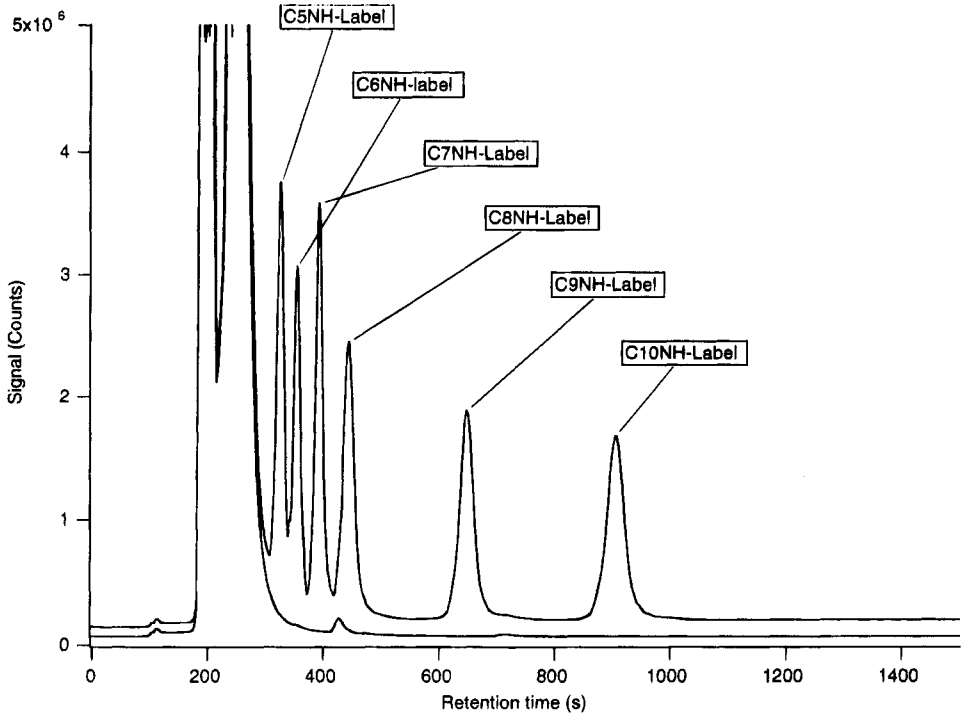

Figure 3. LC chromatograms of a mixture of primary amines (upper trace) and a blank (lower trace), both derivatized with CY5.11a-OSuc, using diode LIF detection. Eluent was acetonitrile–water (70/30) + 0.1% trichloroacetic acid.

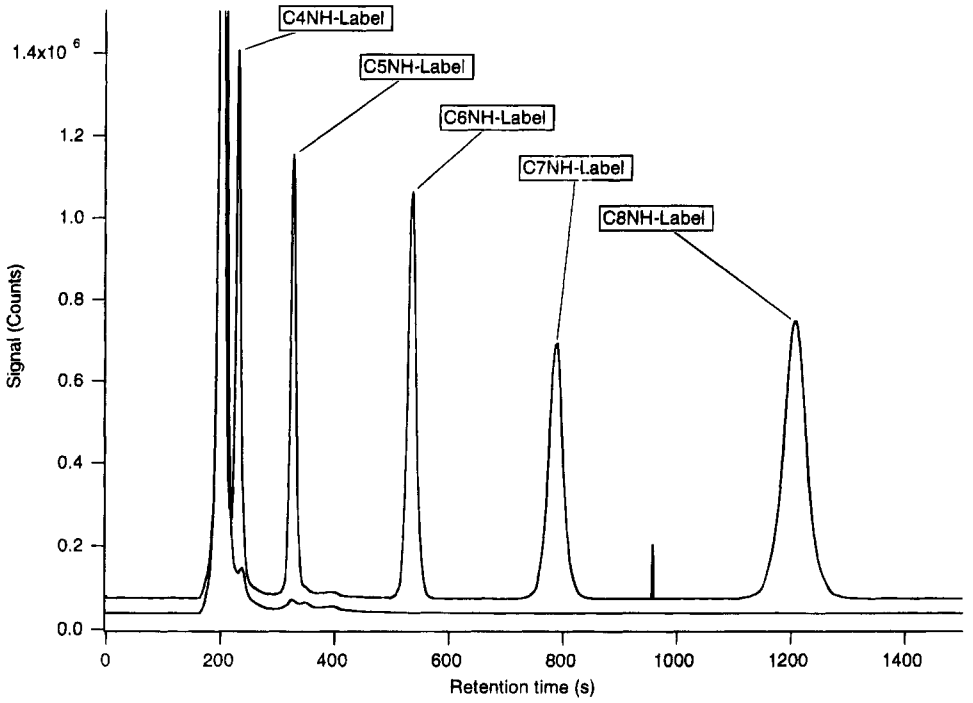

Figure 4. LC chromatograms of a mixture of primary amines (upper trace) and a blank (lower trace), both derivatized with CY5.29-OSuc, using diode LIF detection. Eluent was water–acetonitrile (70/30) + 0.1% trichloroacetic acid.

concentration of 1×10^{-6} M. In other words, the detection of *n*-octylamine and the other alkylamines is limited by the derivatization step.

Obviously, the dimension of the chromatographic window for the derivatives is limited by the labeling agent. We therefore studied squaraine-1-OSuc and CY5.29-OSuc in addition to CY5.11a-OSuc. Chromatographic data for several primary and secondary amine derivatives are included in Table 4. Except for the increase in relative retention of the derivatives compared to the labeling agent, the labeling characteristics are the same as for CY5.11a-OSuc. Figures 3 and 4 display the chromatograms of a mixture of derivatized primary amines using CY5.11a-OSuc and CY5.29-OSuc, respectively. The solutions containing the derivatized amines were diluted 5000-fold before LC–diode LIF analysis.

The chromatograms show that separation of small alkylamines is possible, despite the fact that the label is by far the larger part of the derivative formed. Especially for the labels containing two sulfonate substituents (like CY5.29-OSuc), efficient separation of short-chain amines from the unreacted label is achieved. Simultaneous analysis of long-chain amines requires a step gradient to

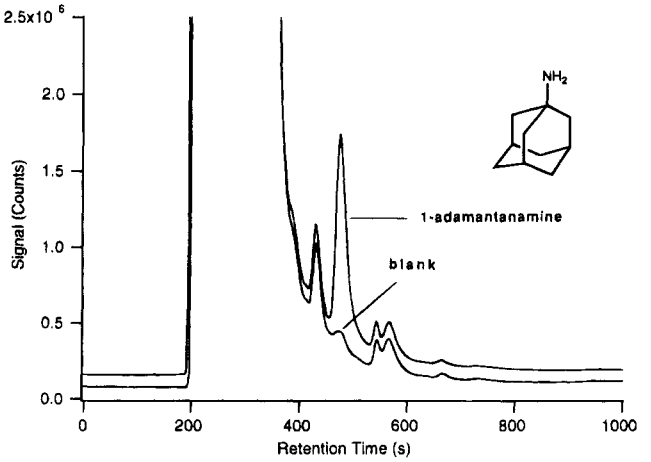

Figure 5. LC chromatograms of blank urine (lower trace) and urine spiked with 5×10^{-7} M 1-adamantanamine using an offset of 0.1×10^6 counts (upper trace), both derivatized with CY5.11a-OSuc, using diode LIF detection. For details, see text. Eluent was acetonitrile–water (70/30) + 0.1% trichloroacetic acid.

higher acetonitrile percentages, i.e., 70%.

The primary amines have a reactivity similar to that of *n*-octylamine, so for all primary amines, the optimum reaction conditions could be used as elucidated for *n*-octylamine. In line with literature reports on the labeling of amines with succinimidyl esters, under the experimental conditions applied, secondary amines react somewhat slower. For these amines, the reaction time had to be prolonged to 1 h for the derivatization to be complete at analyte concentrations below 5×10^{-7} M. It should be noted that the spectroscopic properties of the labels and the derivatized analytes differ only marginally, even if there is no methylene bridge between the carboxylic group and the fluorophore. As a result, quantification of the derivatives is possible on the basis of their peak areas.

To show the applicability of the present procedure for biological samples, morning urine of a healthy volunteer was spiked with 1-adamantanamine at a clinically relevant concentration of 5.0×10^{-7} M. The urine was cooled to 4 °C immediately after collection and filtered through a FP030/3 disposable cellulose acetate filter (0.2-μm pore diameter) (Schleicher & Schuell) before further treatment. The cleanup of a spiked and a blank urine sample was performed by extraction according to the procedure of Sioufi et al.[16] To 500 μL of sample were added 500 μL of 1 M sodium hydroxide and 1 mL of toluene. The tubes were shaken mechanically for 10 min and centrifuged at 3000*g* for 5 min. A 500 μL aliquot of the toluene phase was transferred to another tube, and the solvent was evaporated. The sample was redissolved in 500 μL of 5×10^{-5} M CY5.11a-OSuc in acetonitrile, and the derivatization was performed under the same conditions as for *n*-octylamine. The chromatograms obtained for the blank and spiked urine samples are shown in Figure 5, using the same chromatographic system as for the separation of the *n*-alkylamines. It is clear that 1-adamantanamine can easily be detected at this concentration level. The other peaks in the chromatograms indicate the presence of other amines in urine because these did not show up if distilled water was used instead of urine. The extraction recovery of 1-adamantanamine was 96 ± 7% (±SD, $n = 6$), as was determined by comparing samples obtained by extraction and directly spiked samples (both in acetonitrile). The injection and reaction repeatability ($n = 6$) in urine were 1.5% and 5.2%, respectively.

CONCLUSIONS

The applicability range of diode laser-induced fluorescence detection in liquid chromatography has been significantly extended by the development of a chemical derivatization procedure for amines. Among the three types of near-IR fluorophores studied, oxazines/thiazines, squaraines, and dicarbocyanines, the latter two types were successfully converted into labels containing a succinimidyl ester group. These labels show an acceptable fluorescence quantum yield ($\Phi_f = 0.1–0.3$) in reversed-phase LC solvent mixtures. They can be used for covalent labeling of both primary and secondary amines, typical conditions being a reaction time of 45–60 min using 0.5% triethylamine and a reaction temperature of 60 °C. The labels that contain two sulfonate substituents allow an efficient separation of derivatized short-chain amines from the unreacted label.

Derivatization is best performed in pure acetonitrile (with triethylamine as an activating base) in order to prevent hydrolysis of the label. In practice, the interfering role of excess label during LC analysis can be reduced if the label is hydrolyzed by addition of an alkaline buffer after labeling of the analytes; the carboxylic acid obtained elutes much earlier than the succinimidyl ester label itself.

It has also been observed in other studies on derivatization that the real-life detection limit is determined by the reactivity of the labeling agent. For *n*-octylamine, a difference of 4 orders of magnitude exists between the actual detection limit (2×10^{-8} M) and the detection limit of 2×10^{-12} M obtained by diluting a solution derivatized at a rather high concentration. The latter value, which corresponds to an injected amount of 50 amol of analyte, clearly demonstrates the high sensitivity of LC–diode LIF detection. The applicability of the method to the analysis of amines in biological samples is shown by the determination of 5×10^{-7} M 1-adamantanamine in spiked urine.

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