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

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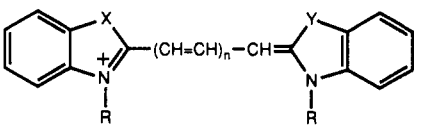
New cyanine labels containing only a single active group for the chemical derivatization of thiols were synthesized in order to apply visible diode laser induced fluorescence (DIO-LIF) detection in conventional-size liquid chromatography (LC). 2-Mercaptobenzothiazole (MBT) was selected as test compound. The most appropriate label was CY5.4a-IA, a sulfonated dicarbocyanine with a iodoacetamide reactive group. The detection limit of labeled MBT in the LC-DIO-LIF system was 8 $\times 10^{-12}$ M. The detection limit of MBT was 1×10^{-9} M; lower concentrations are not labeled quantitatively. The injection repeatability was 1.0% (n = 6), the reaction repeatability was 3.0% (n = 6), and linearity was observed from 2.5×10^{-9} to 1.0×10^{-5} M. As an example, MBT-spiked river water and urine samples were derivatized with CY5.4a-IA and analyzed by LC-DIO-LIF. Hardly any interference was found. The method compares very favorably with existing techniques.

INTRODUCTION

Diode laser induced fluorescence (DIO-LIF) detection has several advantages over conventional LIF detection using argon ion or helium–cadmium lasers:[1] (i) because of the long wavelengths provided by diode lasers (>630 nm), background interferences of the matrix are strongly reduced, (ii) the laser output power can be stabilized to ~0.01%, which results in a significantly decreased flicker noise, (iii) the Raman interference, although extended over a broader wavelength region, is diminished since the scatter intensity is proportional to λ^{-4} (λ being the wavelength), and (iv) diode lasers are cheap and can have lifetimes of over 50 000 h. There is, however, also a serious limitation of DIO-LIF detection. The number of analytes possessing native fluorescence in the red or near-infrared (IR) region of the spectrum is limited, which means that only a few compounds can be detected directly with DIO-LIF.[2] As a result a reaction–detection procedure—using covalently bound fluorophores—will normally be required for DIO-LIF detection. Fluorophores that in principle can be used in the wavelength area are thiazine-, oxazine-, and cyanine-type compounds.[1]

Several oxazines and thiazines contain primary or secondary amino groups, which can be applied for labeling of carboxylic acids via bifunctional carbodiimides.[3] An example is the covalent labeling of albumin with Oxazine 750 resulting in a detection limit of 0.13 pmol.[4] Arginine and glycine can be coupled with an Azure B-derived label. Using a capillary zone electrophoretic separation, a detection limit of 10 pmol was obtained after dilution of a concentrated sample.[2]



X, Y = O, S, $C(CH_3)_2$ or CH=CH

n = 0 - 4 and R = $(CH_2)_x$-CH_3 or $(CH_2)_x$-SO_3^-

Figure 1. Basic chemical structure of cyanines.

Cyanines (Figure 1) possess absorption bands between 600 and 900 nm with high molar absorptivities (ϵ > 100 000 L cm^{-1} mol^{-1}) and are strongly fluorescent.[5] Furthermore, their excitation spectra can be rather easily influenced by changing the structure of the compound. Red shifts in the excitation maxima of ~100 nm are effected by incorporating one additional CH=CH entity in the polymethine chain: if for a certain dicarbocyanine (n = 2, cf. Figure 1) the excitation maximum is at 680 nm, a similar tricarbocyanine (n = 3, cf. Figure 1) will have its excitation maximum at ~780 nm. Smaller shifts can be achieved by changing the substituents on the aromatic or heterocyclic rings. Last but not least, the water solubility can be influenced by applying different N-substituents. The possibility of using cyanine derivatives for covalent labeling was recently reviewed by Patonay and Antoine.[5] Compounds with isothiocyanate, iodoacetamide, and *N*-succinimidyl as active groups have been synthesized by Waggoner et al.[6]

Several examples of DIO-LIF detection with cyanines as fluorescent probes have been described.[4] In most cases an ion-pair extraction system is used.[7] Positively charged cyanines form ion pairs with negatively charged molecules (i.e., surfactants), and the fluorescence of the ion pair is detected after separation from the excess of reagent.[8] Determination limits of 10^{-7} M have been obtained for a surfactant with NK427, a positively charged cyanine, for which a detection limit of 5.0×10^{-12} M was obtained. Another example is based on the physical adsorption of Indocyanine Green (ICG) to serum albumin. After separation on a gel filtration column, the detection limit is ca. 1.5 pmol.[9] Unfortunately, the available fluorophoric agents are not

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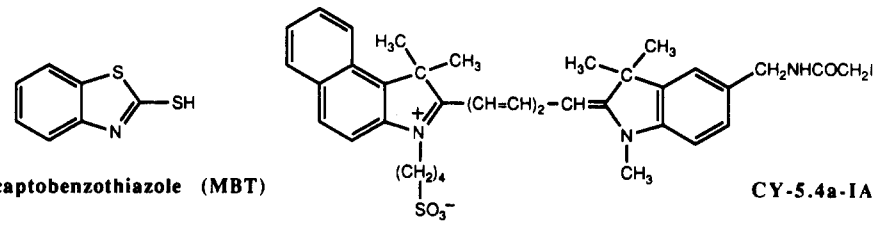

Figure 2. Chemical structures of 2-mercaptobenzothiazole (MBT) and CY5.4a-IA.

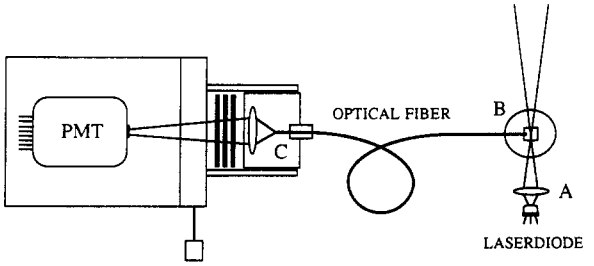

Figure 3. Light path in DIO-LIF detection system: (A) excitation light from diode laser to flow cell; (B) collection of emission light with optical fiber; (C) focussing of collected light on photomultiplier tube (PMT).

appropriate for covalent labeling of trace level analytes. Generally, their purity is insufficient and, furthermore, they do not contain the chemical functionalities required for the derivatization reaction.

In this paper the applicability of precolumn derivatization for DIO-LIF detection in column liquid chromatography (LC) is described. Cyanines containing a iodoacetamido group are used for the covalent labeling of thiol-containing compounds. These solutes possess high fluorescence quantum efficiencies in aqueous solutions, which is rather important, since reversed-phase LC (RP-LC) is the most frequently applied mode of LC.

The test compound in this study is 2-mercaptobenzothiazole (MBT); its structure is depicted in Figure 2. MBT and its derivatives are used as fungicides, bactericides, disinfectants, and wood preservatives.[10,11] In addition, MBT and its corresponding sulfonamides are applied as rubber vulcanization accelerators.[12] Since there is a real danger for human beings to be exposed to MBT, the U.S. Environmental Protection Agency as well as the German Advisory Committee on Existing Chemicals has recommended that it be evaluated for potential hazards.[13] This means that a selective and sensitive analytical method for the determination of MBT and its main metabolites (e.g., thioglucuronide) is required.[14]

EXPERIMENTAL SECTION

Apparatus. For the detection, a diode laser induced fluorescence detection system was developed. The light path in the detection unit is shown in Figure 3. Excitation was provided by a LAS200-670-10 diode laser (Lasermax, Rochester, NY) delivering 9.5 mW at 670 nm, focused at a distance of 10 cm. A homemade quartz flow cell (Suprasil I) with external dimensions of 4.0 × 4.0 × 10 mm and an internal bore of 1.1 mm was used. Collection of the fluorescence light was performed via a 600-μm (core diameter) quartz fiber (Quartz & Silice, Uithoorn, The Netherlands), which was put into the internal bore of the flow cell.[15] The diode laser was positioned on the same optical bench as the detector cell at a distance of ~10.5 cm. Because of the elliptical profile of the beam emitted by the diode laser, the beam was orientated with the longer side perpendicular to the flow direction. The distance between the beam and the fiber tip was 2.0 mm. The analytical volume illuminated by the diode laser is 1.0 μL. The fluorescence light was guided through the fiber to an adjustable lens holder, focused through a 2.5-cm lens (*f*/1) and three Schott RG695 filters onto a Model C-31034 GaAs photomultiplier (PMT; RCA, Lancaster, UK). Focusing is necessary because of the small active area of the PMT (7.6 × 23 mm, rectangular) and the large numerical aperture of the fibers used. The PMT was operated at 1750 V with an EG&G Ortec (Oak Ridge, TN) Model 456H high-voltage power supply and cooled to −15 °C with a PMT housing from Products for Research (Danvers, MA), regulated with a homemade controller. A Thorn EMI Gencom Inc. (Fairfield, NJ) C-10 photoncounter was used with a time constant of 1 s for data processing.

Reagents. HPLC-grade solvents were purchased from J. T. Baker Chemicals (Deventer, The Netherlands) and applied as such, except for methanol, which was distilled before use. Water was demineralized using a Milli-Q (Millipore, Bedford, MA) water purification system. 2-Mercaptobenzothiazole was obtained from Merck (Darmstadt, Germany). Care should be taken when this compound is handled. Contact with skin should be avoided, and the dust should not be breathed. 1,3,3-Trimethoxypropene was supplied by Kodak (Weesp, The Netherlands). All other chemicals were obtained from Aldrich (Beerse, Belgium) at the highest purity available. Urine samples were filtered over a 0.2-μm FP030/30 disposable cellulose acetate filter (Schleicher & Schuell, Dassel, Germany) before derivatization took place.

Procedures. Synthesis of the Derivatization Labels. *Starting Compounds.* The starting compounds I and II (Figure 4) for the derivatization labels were synthesized from butane 1,4-sultone and 2,3,3-trimethyl-(3*H*)-indole and butane 1,4-sultone and 1,1,2-trimethyl-(1*H*)-benz[*e*]indole, respectively, according to standard methods. 5-(Chloroacetamido)-1,3,3-trimethyl-2-methyleneindoline (III), and 5-[(chloroacetamido)-methyl]-1,3,3-trimethyl-2-methyleneindoline (IV) were prepared as described by Gale and Wilshire.[16] Several cyanine labels were synthesized using these starting compounds, according to two procedures (A and B). A method similar to procedure A has been described by Ernst et al. for the synthesis of CY5.3-IA, CY5.4-IA, and CY7.4-IA (Table I).[17]

Procedure A. In order to synthesize dicarbocyanines, 3.0 mmol of I or II was added to 3.0 mmol of V (see Figure 4) in 10 mL of acetic anhydride and heated at 125 °C for 30 min. For the synthesis of tricarbocyanines, V was replaced by VI. The solution of the anil formed was cooled to room temperature, and either 2.5 mmol of III or IV was added. The resulting mixture was heated to 120 °C for 20 min, cooled on ice, and diluted with 150 mL of diethyl ether. The precipitate was collected and purified over a column containing 85 g of silica gel (70–100 mesh; Merck). Elution was performed with a chloroform–methanol gradient starting with 100 ml of 5% methanol (v/v) and increasing the methanol concentration by 5% every 100 mL. All products were eluted when 30% methanol (v/v) was reached. The product was refluxed for 4 h with a 10-fold excess of sodium iodide in 50 mL of methanol, after which the solvent was removed by evaporation under reduced pressure. The resulting solid was redissolved in 20 mL of methanol and cooled with ice. After filtration, the label was precipitated by the addition of 100 mL of diethyl ether and washed with 20 mL of chloroform–hexane (50/50, v/v).

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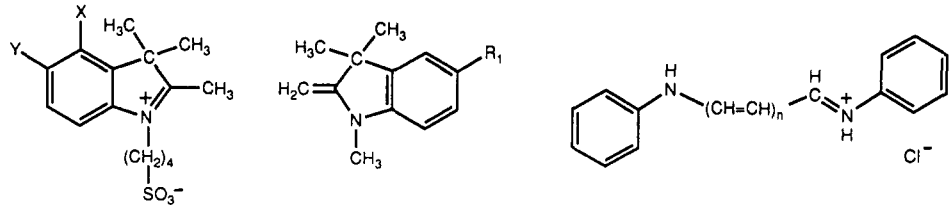

(I) X,Y = H,H
(II) X,Y = -C=C-C=C-
(III) R_1 = $NHCOCH_2Cl$
(IV) R_1 = $CH_2NHCOCH_2Cl$
(V) n = 1;
(VI) n = 2;
(VII) $(CH_3O)_2CHCH=CHOCH_3$

Figure 4. Starting compounds for the synthesis of cyanine derivatives.

Table I. Structure Elements and Spectroscopic Parameters of Cyanine Labels

	structure elements[a]				$\lambda_{ex,max}$	ϵ_{max}[b]	$\lambda_{em,max}$	
compd	n	X	Y	R_1	(nm)	($\times 10^{-3}$)	(nm)	Φ_f[c]
CY5.3-IA[d]	2	I	I	IA	662	197	689	0.12
CY5.3a-IA	2	I	II	IA	676	256	704	0.12
CY5.4-IA[d]	2	I	I	$IACH_2$	652	128	669	0.27
CY5.4a-IA	2	I	II	$IACH_2$	665	139	689	0.25
CY7.4-IA[d]	3	I	I	$IACH_2$	752	195	790[e]	
CY7.4a-IA	3	I	I	IA	756	210	803[e]	

[a] I and II are the structure elements as shown in Figure 4. IA, iodoacetamido; $IACH_2$, iodoacetamidomethyl. [b] Molar absorptivity in L mol^{-1} cm^{-1}. [c] Fluorescence quantum yield. [d] Values are taken from ref 16. [e] Determined with a R928 PMT, which is less sensitive at wavelengths beyond 780 nm; this probably influenced the position of the observed maxima.

Table II. R_f Values of Cyanine Labels and Corresponding L-Cysteine and MBT Derivatives

	R_f values				
	silica[a]		C-8[b]		
label	label	cysteine deriv	label	MBT deriv	LOD[c]
CY5.3-IA	0.37	0.02	0.36	0.16	3.5×10^{-11}
CY5.3a-IA	0.38	0.03	0.39	0.18	1.5×10^{-11}
CY5.4-IA	0.40	0.03	0.38	0.22	6.7×10^{-11}
CY5.4a-IA	0.37	0.02	0.42	0.25	8.3×10^{-12}
CY7.4-IA	0.52	0.03	0.31	0.11	2.9×10^{-10}
CY74.a-IA	0.51	0.03	0.31	0.12	2.3×10^{-10}

[a] Eluent, ethanol–acetic acid (95/5, v/v). [b] Eluent, methanol–water (75/25, v/v). [c] Limit of detection for the MBT derivative (mol/L).

The labels synthesized with this procedure seemed to be pure when checked with thin-layer chromatography (TLC) using silica gel (Merck) plates and eluting with either acetone–ethanol (80/20, v/v) or chloroform–methanol (70/30, v/v). However, using RP-LC on a 250 × 30 mm i.d. slurry-packed C-18 column (LiChrosorb RP-18, 5 μm, Merck) and methanol–water (65/35, v/v) as the eluent, all products were shown to be composed of several compounds.

Procedure B. This method was used as an alternative for the synthesis of the dicarbocyanines. A mixture of 0.2 mmol of I or II and 0.9 mmol of VII in 1 mL of concentrated acetic acid was stirred for 30 min at room temperature. The yellow-brown mixture was diluted with 10 mL of anhydrous diethyl ether and cooled in an ice bath. A yellow-green gum separated, of which the supernatant was removed by decantation. The gum was dissolved in 5 mL of methanol, followed by a fast addition of 25 mL of cold diethyl ether, resulting in the precipitation of a green powder, which was collected on a paper filter. To 0.15 mmol of the resulting product was added an equivalent amount of III or IV in just enough methanol to dissolve both compounds. The mixture was stirred for 24 h. After being cooled in ice, the product was isolated by decantation of the methanol and repeatedly washed with diethyl ether. The product was dissolved in 10 mL of methanol and refluxed for 4 h in the presence of a 10-fold excess of sodium iodide. After cooling to room temperature, the methanol was removed under reduced pressure. Reprecipitation with diethyl ether, after redissolvation in methanol, gave the desired label. All labels synthesized using procedure B seemed to be pure by RP-TLC with C-8 (Merck) material as the stationary phase and chloroform–methanol (70/30, v/v) as the mobile phase. When injected on a C-18 column (see procedure A) and eluted with methanol–water (70/30, v/v), the total amount of impurities appeared to be less than 1%. The di- and tricarbocyanine labels are stable solids. However, solutions of tricarbocyanines were sensitive to heat and light. Solutions of dicarbocyanines were stable for over 3 months when kept in the dark at room temperature. To ensure the quality of all derivatization labels, solutions of both di- and tricarbocyanines were kept in the dark at 4 °C before use.

Spectrophotometric and Chromatographic Characterization. UV–visible absorption spectra of the labels were recorded on a DU-64 spectrophotometer (Beckman, Anaheim, CA), and fluorescence emission spectra on a LS-50 spectrofluorometer (Perkin-Elmer Nederland B. V., Gouda, The Netherlands) equipped with a R928 photomultiplier tube (Hamamatsu Photonics, Hamamatsu City, Japan). Determination of the fluorescence quantum yield was performed as described by Ernst et al.[17] To characterize the properties and the reactivity of the labels, L-cysteine and MBT were derivatized using a published procedure.[17] Derivatization was performed using 5.0×10^{-4} M of both the label and the analyte, in methanol during 2 h at room temperature.

TLC of the labels and the L-cysteine derivatives was performed on silica gel 60 (Merck) with ethanol–concentrated acetic acid (95/5, v/v) as the eluent. R_f values of the labels and the MBT derivatives were also determined with RP (C-8) TLC (Merck) using methanol–water (75/25, v/v) as the eluent (Table II).

Derivatization. In order to investigate quantitative aspects of the MBT derivatization, a stock solution of MBT was prepared by diluting a 10^{-3} M solution in methanol–water (50/50, v/v) containing 1 mM Na_2EDTA 100 times with methanol–water (90/10, v/v). The stock solution was stored in the dark and freshly prepared every week. Derivatizations were performed within 2 h after dilution of this solution. A 500-μL aliquot of a 5.0×10^{-5} M solution of purified CY5.4a-IA in methanol–water (70/30, v/v) was added to 500 μL of the MBT solution and bubbled with dry nitrogen for 1 min. Subsequently, the reaction vial was closed and the mixture was heated for 1.5 h at 65 °C. Finally, 25 μL of the reaction mixture, diluted in order to achieve a final concentration between 10^{-8} and 10^{-10} M, was injected onto a RP-LC system.

Liquid Chromatography. Isocratic elution of the MBT derivative and the nonreacted label was performed with methanol–10 mM phosphate buffer (pH 6.8; 65/35, v/v) containing 1 mM triethylamine. The mobile phase was delivered at a rate of 0.75 mL/min by a Model 300 high-precision pump (Gynkotek, Germering-München, Germany). A Valco injection valve, equipped with a 25-μL loop, was used for injection of the samples on a 150 × 3.1 mm i.d. analytical column, slurry packed with 5-μm LiChrosorb RP-8 (Merck) particles. Absorbance detection of MBT was performed with a HP 1050 absorbance detector (Hewlett-Packard, Waldbronn, Germany) after separation on a 250 × 3.1 mm i.d. C-18 (5-μm particles) Vydac (Hersperia, CA) analytical column using acetonitrile–water–phosphoric acid (30/69.9/0.1 v/v) as the mobile phase.[18]

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RESULTS AND DISCUSSION

Cyanine Labels for Precolumn Derivatization. In contrast to most of the cyanine labels described so far which contain two active groups, in the present study compounds were synthesized with only a single active group. This approach was followed in order to avoid multilabeling reactions, which can hinder detection of low analyte concentrations, especially in complex matrices. To increase the solubility and to reduce aggregation of this type of fluorophore in water, sulfonate substituents can be attached. In this case only one sulfonate substituent was incorporated, as purification of the cyanine labels is more difficult when sulfonate substituents are present at both sides of the polymethine chain. Several dicarbo- and tricarbocyanine labels with iodoacetamido reactive groups for selective derivatization of thiols were synthesized.

In order to obtain optimum excitation with visible diode lasers (665–675 nm), the chemical structure of existing labels had to be modified (Table I). Replacing an indole substituent without a reactive group by a benz[*e*]indole substituent (compare I and II in Figure 4) resulted in a bathochromic absorption shift allowing optimal use of the 670-nm emission wavelength of commercial diode lasers. This effect can be seen by comparing the absorption maxima of compounds CY5.3-IA and CY5.3a-IA in Table I.

The iodoacetamido reactive group was coupled to the aromatic part of the cyanines, either directly or by using a spacer. Direct linking causes an extended conjugation of the fluorophore with the amide, which results in a bathochromic shift in the absorption maximum of ~10 nm and a somewhat larger shift in the emission maximum. Coupling via an alkyl substituent induces only small shifts in both the absorption and emission maxima. Although, at first sight, direct linking seems to be advantageous because of the larger Stokes shift for CY5.3-IA and CY5.3a-IA, a significantly lower fluorescence quantum yield was observed for these compounds when compared with CY5.4-IA and CY5.4a-IA (Table I). Furthermore, the still relatively small Stokes shift in dicarbocyanine labels makes it difficult to separate the emission and excitation light with simple cutoff filters. Therefore, in addition, some tricarbocyanines were synthesized. Although excitation at 670 nm is not optimal for these compounds, selective detection of fluorescence is relatively simple as the emission maxima lie beyond 750 nm. Disadvantages of most tricarbocyanines, compared with the dicarbocyanines, are their lower chemical and thermal stability as well as their reduced photostability. Two tricarbocyanines with iodoacetamido reactive groups (CY7.4-IA and CY7.4a-IA; Table I) were found to be reasonably stable and their labeling capacity was tested in the same way as for the dicarbocyanines.

In order to test the potential of these cyanine-type fluorophores in covalent labels, flow injection analysis (FIA) was performed with the DIO-LIF system described and a comparison was made with Rhodamine 800. The FIA detection limit for the CY5.4a-IA label, which was used for labeling in all subsequent experiments if not mentioned otherwise, was 5.0×10^{-13} M, while for Rhodamine 800 a limit of detection of 1.2×10^{-12} M was obtained. This underlines the potential of cyanine labels and also illustrates the good performance of the experimental detection setup. For comparison Imasaka et al. reported a detection limit of 5×10^{-12} M for Rhodamine 800.[4]

Covalent Labeling of Thiols. Several types of thiol-specific labels have been developed over the years, among which were a number of iodoacetamides.[19] Cyanines with iodoacetamido reactive groups have not been used so far for the derivatization of trace amounts of thiols, so that the optimum reaction conditions still had to be determined. This was done by adding 1 mL of 2.5×10^{-5} M CY5.4a-IA, dissolved in methanol–water (70/30, v/v), to a reaction vial. Subsequently the solvent was evaporated and 500 μL of a sample containing 1.0×10^{-7} M MBT was added. Because MBT is present in many rubber-containing products, special precautions had to be taken concerning the reaction vial cappings. No MBT was found in blanks when the reaction vials were closed with crimp-top seals with standard TFE–rubber liners, provided that the vials were covered with PTFE tape before capping. We found that appropriate vials to perform labeling in were hermetically sealable vials supplied by Bester (Amstelveen, The Netherlands). The screw tops of these gas-tight vials are coated with PTFE.

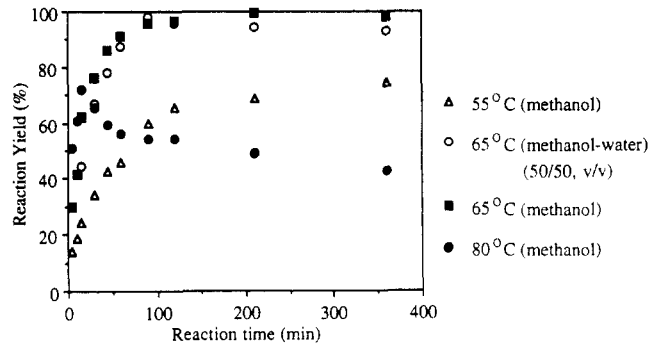

Figure 5. Reaction yield after derivatization of MBT with CY5.4a-IA at different temperatures.

A number of solvents were studied for the derivatization of MBT with cyanines. The best solvents appeared to be methanol and methanol–water (50/50, v/v). Other solvents were not suitable because of insolubility (e.g., hexane) and instability (e.g., acetonitrile) of the cyanines or because of rather long reaction times (e.g., chloroform). The variation in the reaction rate of MBT with the cyanine-type label between pH 6.0 and 8.0 is less than 10%; at 65 °C 500 μL of 1×10^{-7} M MBT is completely derivatized in 50 ± 5 min, which corresponds to an average rate of 1.7×10^{-14} mol/s. However, lower pH are known to result in a reduced reaction rate, while a higher pH is not advisable because of a decreased selectivity of iodoacetamides toward thiols.[20]

In Figure 5, the influence of temperature on the derivatization yield is shown. Although the reaction is relatively fast at 80 °C, rather high concentrations of side products were formed during the derivatization. In both methanol and methanol–water (50/50, v/v), the time to reach the maximum yield is still acceptable at 65 °C (below 2 h) and the formation of side products is no problem when reaction times of less than 4 h are used. The latter temperature was therefore used in further experiments. The major, nonidentified, side product formed after prolonged derivatization (over 4 h at 65 °C) elutes earlier than the nonderivatized CY5.4a-IA and does not interfere with the detection of the MBT derivative.

If low concentrations of MBT are derivatized ($< 5 \times 10^{-9}$ M), the excess CY5.4a-IA is rather high and detection of the thiol derivatives after RP-LC could become difficult because of tailing of the label peak (Figure 6A). The addition of an excess of L-cysteine (5×10^{-4} M) to the reaction mixture, followed by heating to 65 °C for 15 min, allows the removal of the excess of the label. Separation of the MBT derivative and the L-cysteine derivative was performed on a 250 × 3.1

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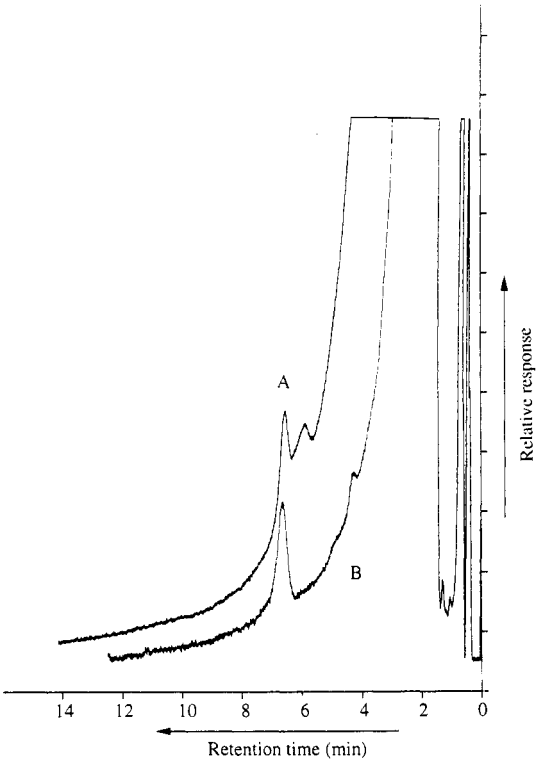

Figure 6. LC chromatograms of derivatization mixtures of MBT with CY5.4a-IA: (A) 50 times diluted sample, originally containing 2.5×10^{-9} M MBT, derivatized with CY5.4a-IA in methanol; (B) same as under A, but after removal of the excess of the label using the purification step based on the reaction with L-cysteine (recorder sensitivity ×2). For further conditions, see text.

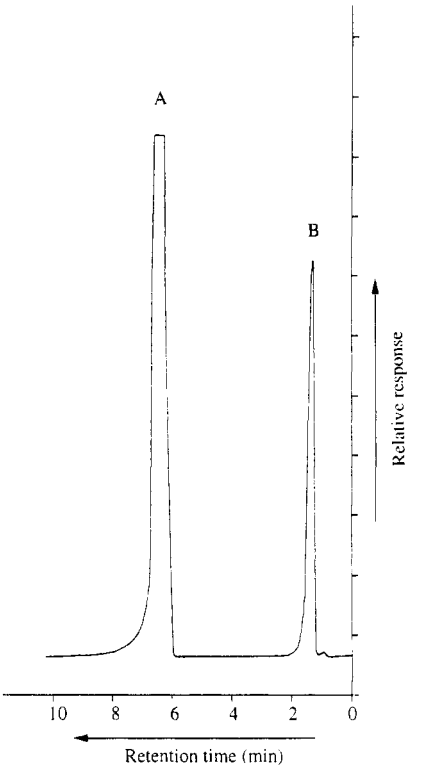

Figure 7. Separation of L-cysteine (A) and MBT derivative (B), as was used for the removal of the excess label from the derivatization product. For further conditions, see text.

mm i.d. homemade slurry-packed silica column (LiChrosorb Si-60, 5 μm, Merck). Methanol–acetic acid–water (94.5/5.0/0.5, v/v) was used as eluent at a flow rate of 1.0 mL/min. When 50 μL of the reaction mixture is injected (dissolved in methanol) on the silica column, the L-cysteine derivative elutes significantly later than the MBT derivative and nonreactive impurities in the labeling agent (Figure 7). Another application of the detection procedure would be the direct detection of physiological concentrations of L-cysteine, but this possibility was not further studied here.

Off-line injection of 25 μL from the first 1.0-mL fraction from the purification column onto the RP-LC system, after dilution with an equivalent amount of water, allowed the detection of MBT concentrations in the original sample down to 1.0×10^{-9} M. A chromatogram of a sample, containing 2.5×10^{-9} M MBT, derivatized and purified as described before is given in Figure 6B. The reaction yield was determined for MBT concentrations of 2.5×10^{-6} and 2.5×10^{-7} M, utilizing a label concentration of 2.5×10^{-5} M [methanol–water (50/50, v/v), 1 h, 65 °C]. After a chromatographic separation the concentration of the MBT derivative in the eluting fraction was determined fluorometrically on the LS-50 spectrometer. It was assumed that the fluorescence quantum yields of the label prior and after derivatization are the same, while it was experimentally determined that both the absorption and emission spectra did not undergo significant changes. For the above concentrations, reaction yields higher than 90% were obtained. Lower MBT concentrations were not tested, but the linearity of the calibration curve (as will be presented below) indicates that the reaction yield is constant, i.e., higher than 90%, down to $\sim 1 \times 10^{-9}$ M. MBT concentrations below 1×10^{9} M resulted in chromatographic peak areas smaller than expected from the calibration curve. Most probably at this concentration level, the derivatization is not complete in 1 h under the experimental conditions at hand, an observation that is well-known from the literature.[21]

The detection limit for MBT is restricted by the derivatization procedure used; the detection limit for the derivatization product of MBT and CY5.4a-IA is 8.3×10^{-12} M (S/N = 3; N taken as peak-to-peak noise), which corresponds with 200 amol of the MBT derivative injected on column. This detection limit is determined by shot-noise and not by fluctuations in the emission of the diode laser. Therefore, future improvements are likely to come from diode lasers emitting more power than presently available. Obviously, the sensitivity of the DIO-LIF system plays no limiting role here. The derivatization reaction inhibits further improvement of the detection limit for MBT below 1×10^{-9} M. The detection limit of the derivative was determined using a 2.5×10^{-9} M solution of MBT in methanol, which was derivatized, purified, and separated according the procedure described above, followed by a 50-fold dilution before injection onto the RP-LC system.

The injection repeatability of the system was determined at a concentration of 1.0×10^{-10} M derivative after labeling 5.0×10^{-8} M MBT ($n = 6$). The repeatability of the reaction procedure was also tested using this concentration ($n = 6$). The relative standard deviations were 1.0% and 3.0%, respectively. A calibration curve showed good linearity after derivatization between 2.5×10^{-9} and 1.0×10^{-5} M.

The equations for the calibration curves are $Y = 2.68\ (\pm 0.02)X - 1.37\ (\pm 34.01)$ ($r^2 = 0.999$) between 5.0×10^{-8} and 1.0×10^{-5} M and $Y = 2.71\ (\pm 0.03)X - 0.27\ (\pm 1.40)$ ($r^2 = 0.999$) between 2.5×10^{-9} and 1.0×10^{-7} M. For both plots seven data points were measured in triplicate; X and Y are the concentration in 10^{-9} M and the relative peak height, respectively.

For concentrations higher than 5.0×10^{-9} M, removal of the label using the reaction with L-cysteine was not a necessity.

(21) Hofstraat, J. W.; Gooijer, C.; Velthorst, N. H. In *Molecular Luminescence Spectroscopy, Part 3*; John Wiley and Sons: New York, 1993; pp 323–443.

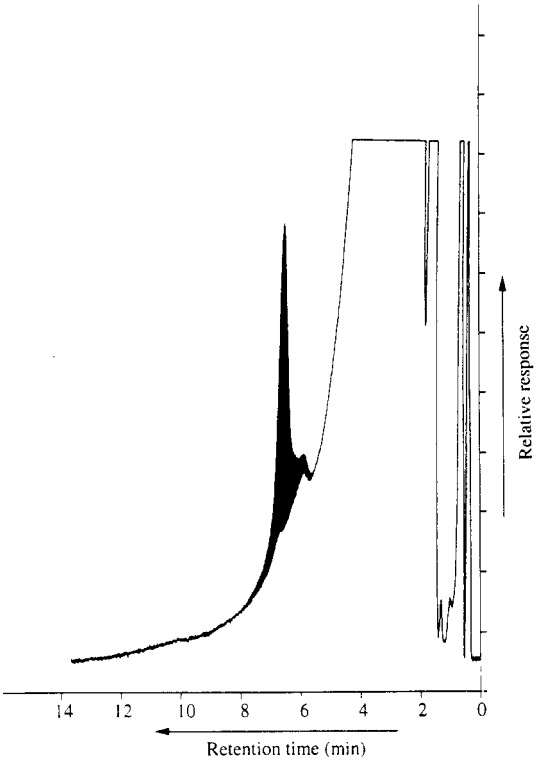

Figure 8. LC chromatograms of Rhine water, blank and spiked with 2.0×10^{-8} M MBT, after derivatization with CY5.4a-IA; both after 100 times dilution. The spike is shown in black.

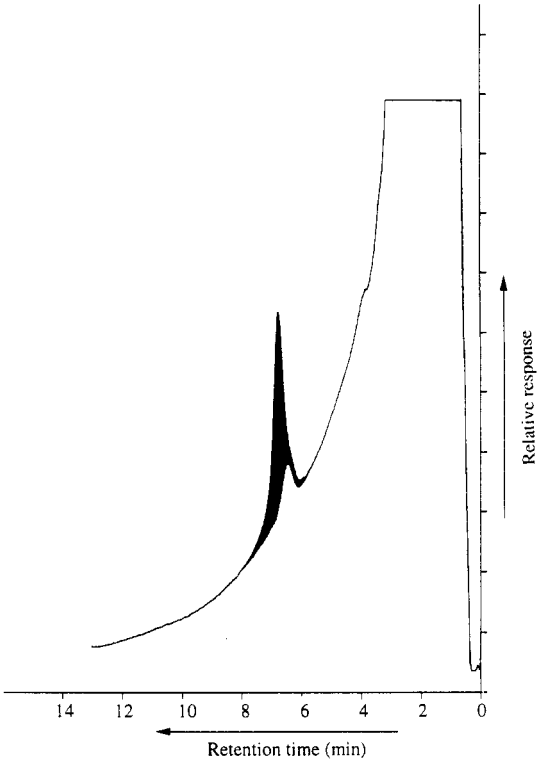

Figure 9. LC chromatograms of urine, blank and spiked with 5.0×10^{-7} M MBT, after derivatization with CY5.4a-IA; both after 500 times dilution. The spike is shown in black.

However, this reaction was helpful with lower MBT concentrations (Figure 6). It would also be advantageous when other thiol derivatives, with retention times shorter than for the MBT derivative, have to be detected.

In addition to L-cysteine and MBT, some other thiols were derivatized as well in order to obtain some insight into the applicability of the described procedure. Using concentrations of 2.5×10^{-5} M for the thiols and 2.5×10^{-5} M CY5.4a-IA and the same reaction conditions as optimized for MBT, quantitative reaction with 2-mercaptoethanol, 2-propanethiol, and 2-aminoethanethiol was observed after RP-LC. For all these (derivatized) compounds the chromatographic characteristics in reversed-phase LC are distinctly different from each other and from the label so that separation is no problem. Only for 2-propanethiol did high label concentrations interfere with the detection of the derivatized analyte. At first sight this chromatographic behavior might be unexpected in view of the bulky derivatizing agent. Presumably the presence of a quaternary ammonium and sulfonate group in the label plays an important role so that the hydrophobic interaction of the derivatized compounds is significantly influenced by the analytes concerned.

Using the reaction conditions optimized for the reaction of CY5.4a-IA with MBT, the detection limit for the MBT derivative of all the labels listed in Table I was determined by diluting a reaction mixture, containing 1.0×10^{-6} M MBT and 2.5×10^{-5} M label. Separation and detection conditions were also applied as optimized for CY5.4a-IA. The results are given in Table II. The relatively poor detection limits for the two CY7 labels are presumably caused by less efficient excitation at 670 nm and lower thermal stability in comparison with the CY5 labels.

Applications. In recent years accidental discharges of MBT polluted several rivers, among them the river Dee (England), which resulted in stopping the drinking water intake from this river. Since gas chromatographic analysis of MBT does not provide satisfactory results because of analyte degradation on the column, a new method, based on dual-electrode electrochemical detection after an LC separation, was developed.[11] For this detection method the standard deviation at 5×10^{-8} M MBT amounts to 1.2×10^{-8} M, while matrix interference is a serious problem.

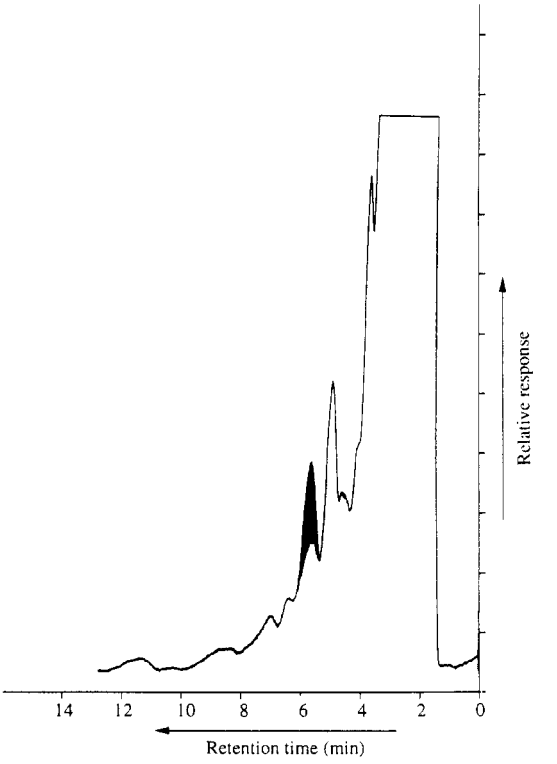

Figure 10. LC chromatograms of urine, blank and spiked with 2.5×10^{-6} M MBT, using UV–visible absorbance detection at 320 nm. The spike is shown in black.

Using the derivatization procedure described, MBT was determined in river Rhine water (Lobith, The Netherlands).

Both a spiked (2.0×10^{-8} M MBT) and a blank sample were labeled with 5.0×10^{-5} M CY5.4a-IA (Figure 8). The injection and derivatization repeatability in Rhine water were 1.0% and 5.9%, respectively. The detection limit for MBT in Rhine water was about the same as in methanol, while no matrix interferences showed up in the blank Rhine water.

The present procedure can also be used for biological matrices such as urine. Morning urine of a healthy volunteer was spiked with MBT and derivatized with CY5.4a-IA. The urine was stabilized with 2 mM Na_2EDTA and cooled (4 °C) immediately after collection. Urine samples were filtered through a FP030/3 disposable cellulose acetate filter (0.2-μm pore diameter) (Schleicher & Schuell) before derivatization was performed. Both a spiked (5.0×10^{-7} M) and a blank sample were derivatized with 5.0×10^{-5} M CY5.4a-IA (Figure 9). Because of the selective wavelengths used for both excitation and emission, hardly any interferences showed up in the chromatograms. Only one small peak was observed close to the MBT derivative peak, probably resulting from derivatization of a nonidentified urinary thiol. If no label was added, injection of a urine sample resulted in a straight baseline, suggesting that this peak is not a result of native fluorescence. Derivatives of other urinary thiols, like L-cysteine, elute very early and do not interfere with the detection of MBT. However, formation of a fluorescent side product of the label cannot be excluded in this complicated matrix.

The above results of LC with DIO-LIF detection of labeled MBT in urine were compared with results of LC of nonlabeled MBT in urine, using absorbance detection. Absorbance detection was performed at 320 nm, the absorbance maximum of MBT ($\epsilon = 30\,000$ L mol^{-1} cm^{-1}). The absorption detection limit of nonlabeled MBT in urine is 5.0×10^{-7} M (Figure 10), which is close to the detection limit of 2.5×10^{-7} M (S/N = 3) in distilled water. In other words, for MBT, precolumn derivatization combined with DIO-LIF detection is far more sensitive, i.e., two decades, despite the fact that the selected test compound has both a relatively long wavelength absorption maximum and a high extinction coefficient.

The results obtained are most promising, indicating that the present procedure can be used for the detection of trace amounts of MBT and other thiols in complex matrices.

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