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Time-resolved luminescence detection of derivatized thiols in column liquid chromatography

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ABSTRACT

The selectivity of luminescence detection in liquid chromatography is enhanced by applying time-resolved detection with a pulsed xenon lamp and a gated photomultiplier, and by making use of labels with a long luminescence decay time (*i.e.*, longer than 0.1 ms). Sensitized Tb^{3+} luminescence, which needs no solvent deoxygenation, was employed in this study. The derivatization of thiol-containing analytes to sensitizing products was studied in batch experiments; in particular, attention was paid to the reaction conditions for the derivatization of glutathione by 4-maleimidylsalicylic acid. In the chromatographic experiments, derivatization was performed pre-column, a Tb^{3+} solution was added post-column to achieve complexation and sensitized Tb^{3+} luminescence was detected. Compared to direct fluorescence detection, sensitized Tb^{3+} luminescence detection gave better results with respect to sensitivity (the limit of detection of glutathione was $2 \cdot 10^{-8}$ *M*, 0.3 ng on column) and, particularly, selectivity, as demonstrated for the spiked urine samples.

INTRODUCTION

Fluorescence spectroscopy offers many possibilities for sensitive and selective detection in liquid chromatography. Detection is based on the native fluorescence of the analyte or, if this is absent or only weak, the analyte may be derivatized to a fluorescent product by a chemical reaction [1]. Although fluorescence detection has an inherent sensitivity, there are several processes which contribute to the background signal, *i.e.*, scattering and fluorescence caused by solvent impurities or an excess of derivatization reagent (which is obviously preferred to be non-fluorescent). These phenomena increase the noise and restrict the achievable detection limits. In time-resolved luminescence spectroscopy, the background originating from these short-lived processes can be excluded by introducing a delay time after the start of the excitation pulse during which the photomultiplier is shut so that the detection of the luminescence takes place after the intensity of the pulse has decayed completely. Long-lived luminescence can be detected very sensitively by gating the photomultiplier during a certain interval after the delay time, as shown in Fig. 1. Time-resolved luminescence detection has already been shown to be an interesting method for use in liquid chromatography for a number of applications [2–5].

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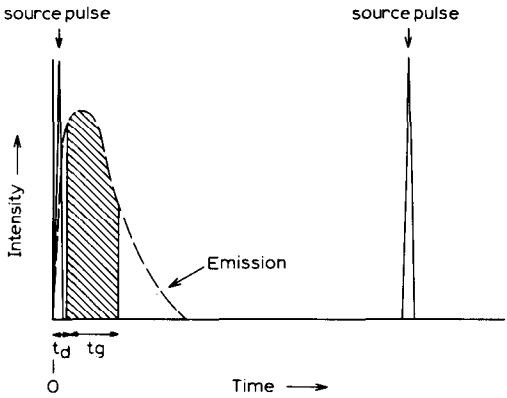

Fig. 1. Emission signal after source excitation flash applying time-resolved luminescence detection: t_d, delay time; t_g, gating time.

Only very few compounds emit long-lived luminescence in liquid solutions. Among these are organic compounds which are phosphorescent in the absence of oxygen and some La^{3+} ions which emit long-lived luminescence ($\tau \geq 0.1$ ms) even in the presence of oxygen [6]. Luminescence of the ions Eu^{3+} and Tb^{3+}, with characteristic decay times of 0.1 and 0.4 ms, respectively, in aqueous solutions, can easily be detected by applying a pulsed xenon lamp. This phenomenom can be utilized for detection purposes in an indirect way, either based on quenching [5,7] or on the sensitizing of lanthanide luminescence, induced by the analytes under consideration. In this paper attention is focused on sensitized luminescence.

Lanthanide ions have low absorptivities (typically smaller than 10 l mol^{-1} cm^{-1}), therefore relatively high concentrations are needed to observe their direct luminescence. Intramolecular sensitizing may occur when the ion forms a complex with an absorbing ligand which, after being excited, transfers its energy to an accepting level of the lanthanide ion [8]. Various compounds are known to have sensitizing properties and can be detected very well by sensitized lanthanide luminiescence detection. For instance, several tetracyclines sensitize Eu^{3+} luminescence [4,9], salicylate sensitizes Tb^{3+} luminesence [10] and some β-diketonates sensitize Eu^{3+} luminescence, whereas others sensitize Tb^{3+} luminescence [8].

To extend the potential of the time-resolved detection of sensitized lanthanide luminescence as a detection method in liquid chromatography, a derivatization reaction has been developed in which non-sensitizing analytes are converted into sensitizing compounds. This is achieved by coupling chromophoric groups, which are known to have sensitizing properties for lanthanide luminescence, to the analyte. Two commercially available derivatization reagents highly specific for thiol groups, *i.e.*, 4-maleimidylsalicylic acid (4-MSA) and 4-iodoacetamidosalicylic acid (4-ISA), were examined. As a salicylate group sensitizes Tb^{3+} luminescence, this ion is used as a long-lived luminophore. Preliminary experiments have shown that 4-maleimidylsalicylic acid has favourable properties for derivatization and detection [11]. In this paper a detailed spectroscopic and chromatographic study is presented, mainly concentrating on gluthathione as a model compound.

EXPERIMENTAL

Chemicals

Terbium(III) chloride hexahydrate (99.9%) was obtained from Aldrich (Milwaukee, WI, U.S.A.), 4-MSA and 4-ISA from Molecular Probes (Eugene, OR, U.S.A.), glutathione (reduced form) from Boehringer (Mannheim, Germany), N-acetylcysteine from Aldrich, L-cysteine, sodium salicylate and Trizma base from Sigma (St. Louis, MO, U.S.A.) and tetrabutylammonium bromide from Kodak (Rochester, NY, U.S.A.). High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Baker (Deventer, The Netherlands).

Instrumentation

Batch experiments were performed with a Perkin-Elmer (Beaconsfield, U.K.) MPF-44 fluorescence spectrometer, supplied with a continuous XBO 150 W xenon lamp and Hamamatsu type R777-01-HA photomultipliers.

The HPLC system consisted of a Gilson (Villiers-le-Bel, France) 302 HPLC pump, a Valco six-port injection valve equipped with a 50-μl injection loop and a stainless-steel column (140 × 3.1 mm I.D.) packed with RoSil (BioRad-RSL, Eke, Belgium) C_{18} HL 5-μm particles. The post-column Tb^{3+} solution was added using a Pye Unicam (Cambridge, U.K.) PU4015 pump. A Perkin-Elmer LS-40 fluorescence detector was used both for fluorescence and for time-resolved Tb^{3+} luminescence detection. Fluorescence detection of the 4-MSA–thiol product was performed at excitation and emission wavelengths of 302 and 408 nm, respectively, whereas the sensitized Tb^{3+} luminescence was detected at excitation and emission wavelengths of 322 and 545 nm, respectively. In the latter mode, the delay and gating times were 0.1 and 2.0 ms, respectively.

Preparation of solutions

Stock solutions of thiols were prepared in doubly distilled water containing $1 \cdot 10^{-4}$ *M* EDTA, acidified with hydrochloric acid (pH 3.0). Stock solutions of 4-MSA and 4-ISA were prepared in ethanol and aqueous Tris buffer, pH 7.5, respectively.

The mobile phase consisted of acetonitrile–water (25:75, v/v), $5 \cdot 10^{-3}$ *M* Tris buffer, pH 7.2, and $1 \cdot 10^{-3}$ *M* tetrabutylammonium bromide; the post-column solution contained 1.10^{-3} *M* $TbCl_3$ in water–acetonitrile (50:50, v/v). The flow-rates of the mobile phase and the post-column solution were both 0.5 ml/min.

The long-term stability of the derivatization products has not been investigated; the derivatized analytes were usually injected within 1 h of preparation.

The clean-up of diluted urine samples was performed using disposable filter holders FP 030/30 2 μm (Schleicher & Schuell, 's-Hertogenbosch, The Netherlands) and 1-ml octadecyl disposable extraction columns, Bakerbond spe 7020-01 (Baker, Phillipsburg, NJ, U.S.A.).

RESULTS AND DISCUSSION

Batch experiments

When choosing a derivatization label, the following aspects have to be considered: the reaction conditions required; the possible occurrence of competitive reactions

TABLE I

DETECTION WAVELENGTHS AND RELATIVE FLUORESCENCE AND SENSITIZED Tb^{3+} LUMINESCENCE INTENSITIES OF SEVERAL SOLUTION

Solutions were prepared in $5 \cdot 10^{-3}$ *M* Tris buffer, pH 7.2; the results in the last column were obtained after the addition of EDTA and sodium hydroxide solution.

Solution	Fluorescence	Sensitized Tb^{3+} luminescence (pH 7.2)	Sensitized Tb^{3+} luminescence (pH 12)
Excitation wavelength (nm)	300–305	320–322	320–322
Emission wavelength (nm)	400–410	545	545
Salicylate	+ + +	+ +	+ + +
4-ISA	+	+	+ +
4-ISA–GSH	+	+	+ +
4-MSA	0	0	0
4-MSA–GSH	+ +	+ +	+[a]

[a] Maximum excitation at 336 nm.

(especially if pre-column derivatization is applied); the difference in spectroscopic properties between the derivatization reagent and its reaction product; and, finally, the linearity, reproducibility and sensitivity of detection of the labelled analytes. Both derivatization reagents studied, 4-ISA and 4-MSA, give fluorescent products on reaction with thiols. The derivatization reaction was first studied in batch experiments by fluorescence spectroscopy using glutathione (GSH) as a model compound. Tb^{3+} was subsequently added and the sensitized lanthanide luminescence was compared with the fluorescence recorded in the absence of Eu^{3+}. The results are discussed below and are summarized in Table I; pure salicylate is included for comparison.

Fluorescence. In a similar manner to other maleimides known from fluorescence derivatization [1,12–18], 4-MSA shows hardly any fluorescence, in contrast with 4-ISA (see Table I). On the addition of GSH to a solution of 4-MSA, the formation of a fluorescent product is observed. In contrast, the presence of a thiol in the 4-ISA solution does not significantly change the fluorescence wavelengths or intensity. The reaction of GSH and 4-MSA was studied in more detail in batch experiments.

Effect of pH on the reaction rate. The reaction rate of the nucleophilic addition of the thiol to the carbon–carbon double bond of the maleimide group is slow in acid solutions and rapid in alkaline solutions as the thiolate ion is a strong nucleophile. As in the following step Tb^{3+} has to be added to the 4-MSA–GSH mixture, the derivatization reaction was studied at pH values where the hydrolysis of Tb^{3+} is relatively slow. In Fig. 2 the increase of fluorescence intensity *versus* time for 4-MSA–GSH at various pH values is shown. It can be concluded that in the buffered solutions the choice of pH is not very critical; at room temperature the reaction is completed within about 10 min. In water (pH 6.0) the reaction proceeds much more slowly, but its rate can be considerably increased by increasing the temperature to 60°C. This is not necessary when the reaction is performed in Tris buffer at pH 7.0–7.5.

Reaction rate at various reagent and thiol concentrations. For a high reaction rate an excess of derivatization reagent is necessary, as shown in Fig. 3. At a 4-MSA concentration of $5 \cdot 10^{-5}$ *M*, a 50-fold excess, the derivatization reaction is completed

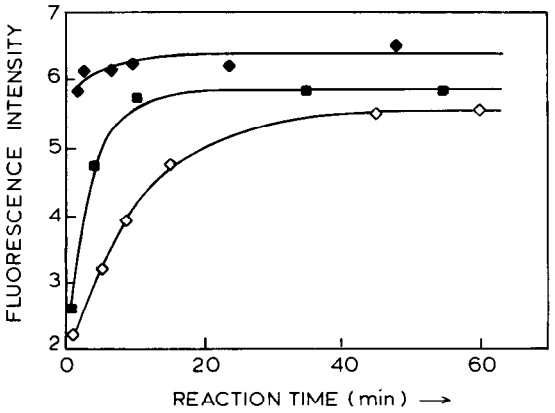

Fig. 2. Reaction rate of 4-MSA and GSH at various pH values: ◇ = water, pH 6; ■ = Tris buffer, pH 7.0; ◆ = Tris buffer, pH 7.5.

in less than 10 min at room temperature. Such an excess is not a serious problem, as the fluorescent 4-MSA–GSH adduct can be readily discriminated from the unreacted, poorly fluorescent 4-MSA. In Fig. 4 the increase of fluorescence intensity is shown for three GSH concentrations at a fixed 4-MSA concentration, indicating the linearity of the fluorescence detection.

Sensitizing of Tb^{3+} luminescence. On the addition of Tb^{3+} to a salicylate, 4-ISA, 4-ISA–GSH, or 4-MSA–GSH solution, the characteristic narrow-banded Tb^{3+} emission becomes clearly visible (see Table I and Fig. 5). Although no time-resolved detection was applied in the batch experiments, the Tb^{3+} luminescence can be easily discriminated from the fluorescence signal as its maximum intensity is at a longer wavelength (545 nm). The appearance of Tb^{3+} luminescence simultaneously involves a decrease in the fluorescence intensity. In 4-ISA and 4-ISA–GSH solutions a rather

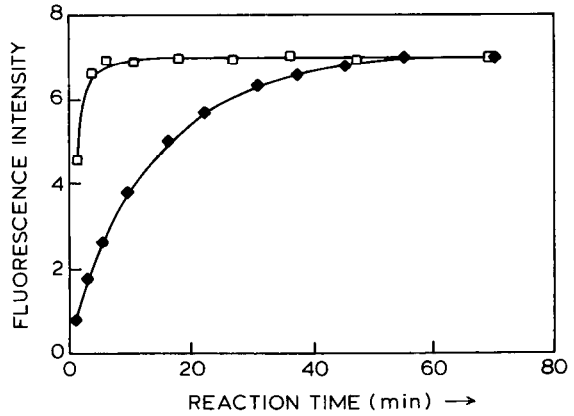

Fig. 3. Reaction rate of 4-MSA and GSH at a five- and fifty-fold excess of 4-MSA. [GSH] $1 \cdot 10^{-6}$ *M*, reaction in Tris buffer, pH 7.0. ◆ = [4-MSA] $5 \cdot 10^{-6}$ *M*; □ = [4-MSA] $5 \cdot 10^{-5}$ *M*.

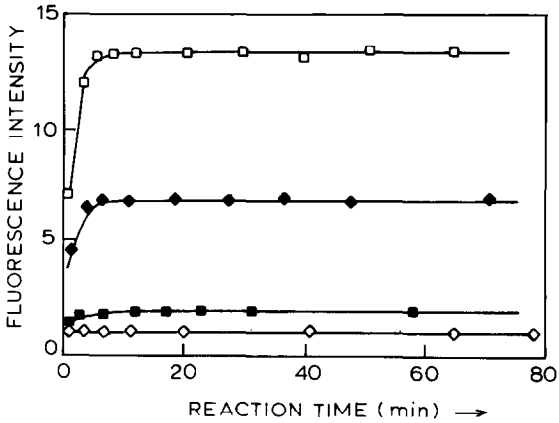

Fig. 4. Reaction rate of 4-MSA and GSH at several GSH concentrations. [4-MSA] $5 \cdot 10^{-5}$ *M*, reaction in Tris buffer, pH 7.0. □ [GSH] $2 \cdot 10^{-6}$ *M*; ◆ = [GSH] $1 \cdot 10^{-6}$ *M*; ■ = [GSH] $2 \cdot 10^{-7}$ *M*; ◇ = blank.

weak sensitized Tb^{3+} luminescence is observed, whereas unreacted 4-MSA does not sensitize the Tb^{3+} luminescence at all.

The intensity of the sensitized lanthanide luminescence as a function of Tb^{3+} concentration (at a fixed concentration of 4-MSA–GSH) is presented in Fig. 6. This figure also shows the decrease of fluorescence noted earlier. More importantly, the direct lanthanide luminescence in the absence of 4-MSA–GSH, which will be present as a background signal and therefore should be as low as possible, is shown. Although around 320 nm the absorptivity of Tb^{3+} is very low [19], at high concentrations some luminescence arising from direct excitation can be observed. A Tb^{3+} concentration of about $5 \cdot 10^{-4}$ *M* is appropriate.

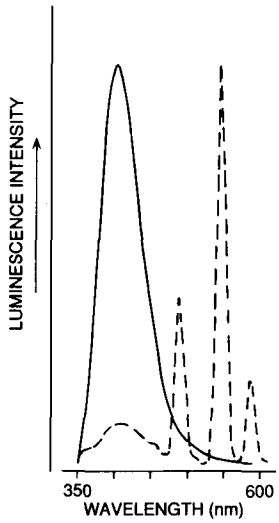

Fig. 5. Fluorescence spectra of 4-MSA–GSH. (——) In the absence of Tb^{3+}, excitation wavelength 302 nm; (--) in the presence of Tb^{3+}, excitation wavelength 322 nm (vertical scale in relative units).

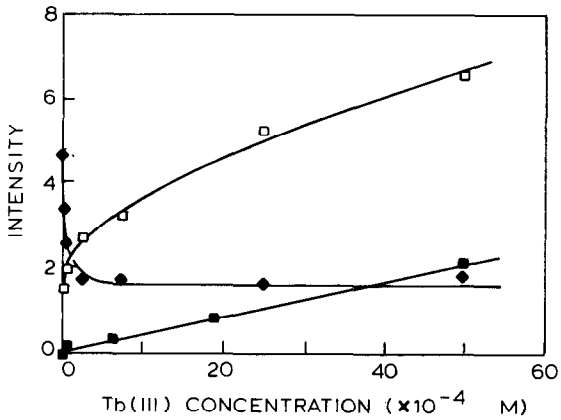

Fig. 6. Luminescence intensities as a function of the Tb^{3+} concentration. □ = Tb^{3+} luminescence intensity sensitized by 4-MSA–GSH, excitation wavelength 322 nm; ◆ = remaining 4-MSA–GSH fluorescence; ■ = direct Tb^{3+} luminescence in the absence of 4-MSA–GSH, excitation wavelength 322 nm.

Tb^{3+} sensitizing at pH ≈ 12. For a salicylate ligand, strong alkaline conditions lead to a better complexation of the lanthanide ion and thus more efficient sensitizing [10]. Tb^{3+} was protected from hydrolysis by a two-fold excess of EDTA before the sodium hydroxide solution was added. As shown in Table I, an enhancement of the Tb^{3+} luminescence intensity as observed for salicylate is not seen for 4-MSA–GSH; the sensitized Tb^{3+} luminescence decreased compared to the signal at a pH of about 7 and the maximum excitation wavelength shifted to 336 nm. In 4-ISA and 4-ISA–GSH solutions the Tb^{3+} luminescence is not constant but increases steadily under these alkaline conditions and becomes very intense after several hours (data are not shown; the intensities presented in Table I were measured within a few minutes of the addition of Tb^{3+} to the solutions).

Concluding remarks. 4-MSA was considered to be the most favourable reagent for these purposes; the reaction is fast, is performed under mild conditions, the detection does not require an extreme pH and, most importantly, the excess of derivatizing reagent, which is necessary for a fast reaction does not interfere with the detection. 4-ISA seems less favourable although the high sensitized Tb^{3+} luminescence intensity at pH 12 may have interesting possibilities. Derivatization with 4-ISA was not further investigated in this work.

Chromatographic results

To apply the detection principle for the thiols described in the preceding section to HPLC, the derivatization of the thiol-containing compound with 4-MSA was performed pre-column. Separation was achieved by ion-pair chromatography using a reversed-phase column; the eluent composition was acetonitrile–water (25:75, v/v) containing $5 \cdot 10^{-3}$ *M* Tris buffer (pH 7.2) and $1 \cdot 10^{-3}$ *M* tetrabutylammonium bromide. The advantage of this chromatographic system over the system described previously [11] in which the separation was performed under acidic conditions is that, instead of two post-column pumps (*i.e.*, one pump to add a buffer to neutralize the effluent to pH 7 and a second pump to add the Tb^{3+} solution), a single post-column

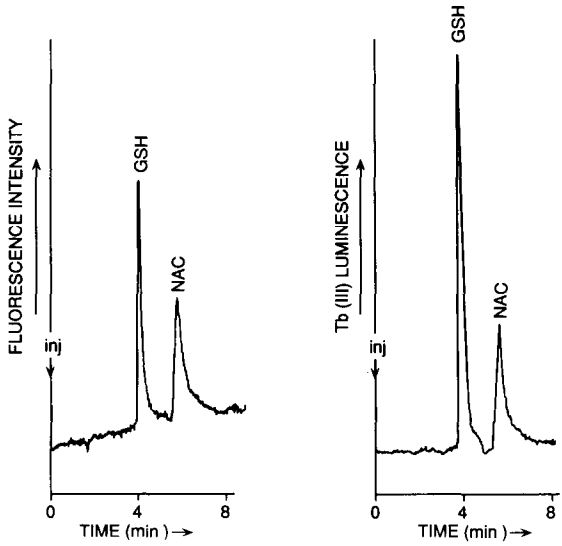

Fig. 7. Mixture of $3.7 \cdot 10^{-7}$ *M* N-acetylcysteine (NAC) and $4.7 \cdot 10^{-7}$ *M* glutathione (GSH) derivatized by 4-MSA ($5 \cdot 10^{-5}$ *M*). Left panel, fluorescence detection (vertical scale in relative units); right panel, sensitized Tb^{3+} luminescence detection (vertical scale extended by two times compared with fluorescence detection).

pump to add the Tb^{3+} solution [$1 \cdot 10^{-3}$ *M* in water–acetonitrile, 50:50 (v/v)] suffices.

The detector used can be operated in a fluorescence mode as well as in a time-resolved (phosphorescence) mode; both methods were applied for the detection of 4-MSA–thiol addition products. In the case of fluorescence detection, no Tb^{3+} solution was added to the effluent as Tb^{3+} quenches the fluorescence. For standard solutions of 4-MSA–GSH it was found that the time-resolved detection mode gave two to three times better signal-to-noise ratios, despite the loss of signal as a result of dilution by the post-column flow. In Fig. 7 chromatograms of a mixture of derivatized N-acetylcysteine and glutathione are shown as obtained by both detection modes.

The detection limit obtained using time-resolved sensitized Tb^{3+} luminescence detection for derivatized glutathione was $2 \cdot 10^{-8}$ *M*, which compares well with the limit of detection for similar thiols obtained by electrochemical methods [20] and by fluorescence detection using other maleimide-based reagents [16,18,21]. However, a few maleimide-based derivatization reagents are reported to give products with considerably lower detection limits [15,17]. It should be noted that these results were obtained for standard solutions. The main advantage of the method reported here is its selectivity. The calibration graph for glutathione was linear from $5 \cdot 10^{-8}$ to $1 \cdot 10^{-6}$ *M* ($r = 0.998$, $n = 8$). In all solutions the 4-MSA concentration was $5.0 \cdot 10^{-5}$ *M* and the reaction time was 10–15 min. The repeatability of the peak heights of an reaction mixture (GSH concentration $1 \cdot 10^{-6}$ *M*) injected four times between 10 and 40 min after preparation was 1% (relative standard deviation).

For L-cysteine, the derivatization procedure gave some stability problems. As noted previously [11], reaction mixtures of this analyte and 4-MSA showed a time-

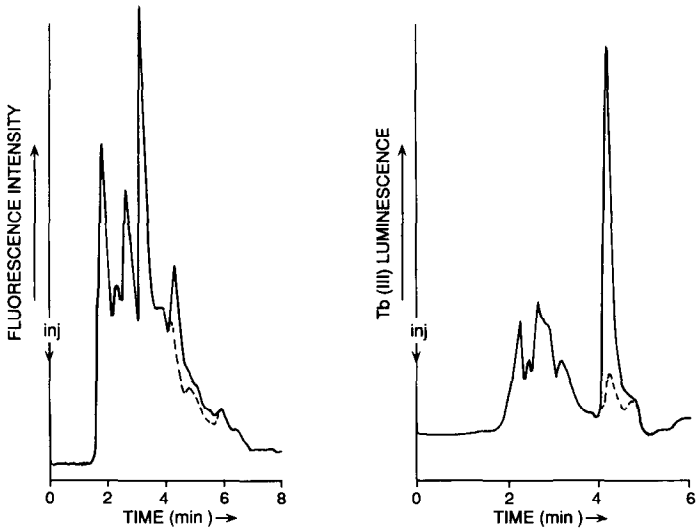

Fig. 8. Chromatograms of a urine sample diluted ten-fold with reaction buffer containing $8 \cdot 10^{-5}$ *M* EDTA and 1.10^{-5} *M* 4-MSA. (--) no GSH added; (——) [GSH] $1 \cdot 10^{-6}$ *M*. Left panel, fluorescence detection; right panel sensitized Tb^{3+} luminescence detection (vertical scales in relative units).

dependent shift in fluorescence wavelengths in batch experiments. These reaction mixtures were also injected in this chromatographic system. On injection of a fresh reaction mixture (10 min), one single peak was observed in the chromatograms. Mixtures injected after standing for more than 1 h at room temperature gave an additional peak at a longer retention time by both fluorescence and sensitized Tb^{3+} luminescence detection. This may be caused by a reaction similar to the thiazane formation described for 1-pyrenemaleimide and cysteine [13].

To illustrate the selectivity of detection by sensitized Tb^{3+} luminescence, the derivatization reaction of GSH was carried out in diluted urine samples as an example of a complex matrix (urine itself does not contain any GSH). To protect the analytical column from contamination, simple clean-up steps were applied before spiking the sample with 4-MSA and GSH. Urine samples, diluted ten-fold with reaction buffer ($5 \cdot 10^{-3}$ *M* Tris buffer, pH 7.0), were filtered over a membrane filter and an octadecyl disposable extraction column. The chromatograms detected by fluorescence and by sensitized Tb^{3+} luminescence are shown in Fig. 8. As a lot of fluorescent compounds are present in urine, the fluorescence detection of 4-MSA–GSH (at these wavelengths) is not very selective. The number of compounds which cause efficient sensitization of Tb^{3+} luminescence is obviously less, although two small peaks interfere with the 4-MSA–GSH peak in the spiked sample. 4-MSA–GSH can be detected far more selectivity by sensitized Tb^{3+} luminescence than by fluorescence detection.

CONCLUSIONS

The results described in this paper show that for thiol functional groups chemical derivatization with 4-MSA is an appropriate procedure to make use of the tempo-

ral resolution in luminescence detection via the post-column addition of Tb^{3+}. On derivatization a product is generated which is able to form a complex with Tb^{3+} and to sensitize its long-lived luminescence. An excess of reagent does not perturb the detection.

The most interesting aspect of the sensitized Tb^{3+} luminescence detection method is the improvement in selectivity compared to fluorescence detection for the analysis of real samples. This is obvious from the experiment performed with spiked urine samples. Whereas in the fluorescence detection mode the derivatized analyte can hardly be seen as it is not separated from other fluorescent components in the sample, in the time-resolved detection mode the signal-to-noise ratio is much more favourable.

Application of this method to the determination of thiols in biological samples is under current investigation. It would also be interesting to follow a similar approach for other function groups.

REFERENCES

1 J. Goto, in H. Lingeman and W. J. M. Underberg (Editors), *Detection-Oriented Derivatization Techniques in Liquid Chromatography*, Marcel Dekker, New York, 1990, p. 323.
2 R. A. Baumann, C. Gooijer, N. H. Velthorst and R. W. Frei, *Anal. Chem.*, 57 (1985) 1815.
3 R. A. Baumann, M. Schreurs, C. Gooijer, N. H. Velthorst and R. W. Frei, *Can. J. Chem.*, 65 (1987) 965.
4 T. J. Wenzel, L. M. Collette, D. T. Dahlen, S. M. Hendrickson and L. W. Yarmaloff, *J. Chromatogr.*, 433 (1988) 149.
5 M. Schreurs, G. W. Somsen, C. Gooijer, N. H. Velthorst and R, W. Frei, *J. Chromatogr.*, 482 (1989) 351.
6 J. L. Kropp and M. W. Windsor, *J. Chem. Phys.*, 42 (1965) 1599.
7 R. A. Baumann, D. A. Kamminga, H. Derlagen, C. Gooijer, N. H. Velthorst and R. W. Frei, *J. Chromatogr.*, 439 (1988) 165.
8 W. R. Dawson, J. L. Kropp and M. W. Windsor, *J. Chem. Phys.*, 45 (1966) 2410.
9 L. M. Hirschy, E. V. Dose and J. D. Winefordner, *Anal. Chim. Acta*, 147 (1983) 311.
10 M. P. Bailey, B. F. Rocks and C. Riley, *Anal. Chim. Acta*, 201 (1987) 335.
11 M. Schreurs, C. Gooijer and N. H. Velthorst, *Anal. Chem.*, 62 (1990) 2051.
12 Y. Kanaoka, M. Machida, K. Ando and T. Sekine, *Biochim. Biophys. Acta*, 207 (1970) 269.
13 C.-W. Wu, L. R. Yarbrough and F. Y.-H. Wu, *Biochemistry*, 15 (1976) 2863.
14 H. Takahashi, Y. Nara and K. Tuzimura, *Agric. Biol. Chem.*, 42 (1978) 769.
15 B. Kågedal and M. Källberg, *J. Chromatogr.*, 229 (1982) 409.
16 J. O. Miners, I. Fearnley, K. J. Smith and D. J. Birkett, *J. Chromatogr.*, 275 (1983) 89.
17 K. Nakashima, C. Umekawa, H. Yashida, S. Nakatsiju and S. Akiyama, *J. Chromatogr.*, 414 (1987) 11.
18 R. A. G. Garcia, L. L. Hirschberger and M. H. Stipanuk, *Anal. Biochem.*, 170 (1988) 432.
19 W. T. Carnall, in K. A. Gschneidner and L. Eyring (Editors), *Handbook on the Chemistry and Physics of Rare Earths*, Vol. 3, North-Holland Publishing Company, Amsterdam, 1979, p. 171.
20 A. J. J. Debets, R. van de Straat, W. H. Voogt, H. Vos, N. P. E. Vermeulen and R. W. Frei, *J. Pharm. Biomed. Anal.*, 6 (1988) 329.
21 M. Johansson and S. Lenngren, *J. Chromatogr.*, 432 (1988) 65.