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Use of enhancers in the HPTLC fluorescence analysis of thiols*

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Abstract: Several thiols of biological and pharmacological interest, including glutathione, coenzyme A, acetylcysteine and captopril were derivatized with the fluorogenic reagents SBD-F and ABD-F and analysed by high-performance thin-layer chromatography (HPTLC)–fluorodensitometry on silica gel 60 plates, using isopropyl ether–methanol–water–acetic acid (9:8:2:1, v/v/v/v) as the developing solvent. The luminescence was considerably increased when several types of enhancers were applied as dipping reagents: Triton X-100, liquid paraffin and cyclodextrins; thus the detectability of the thiol fluorophores was improved. The influence of enhancer concentration, method of application, sample concentration, drying conditions and measuring time after plate dipping were investigated. The greatest enhancement was achieved using a 40% (v/v) solution of Triton X-100 in toluene as a dipping reagent for the determination of SBD–acetylcysteine; more than a 10-fold increase of the fluorescence signal was obtained, allowing low picogram detection limits.

Keywords: *Thiols; HPTLC; fluorescence derivatization; fluorescence enhancement; fluorobenzoxadiazoles.*

Introduction

Fluorimetric analysis is often considered the method of choice for the determination of numerous pharmaceutical and biomedical compounds [1, 2]. Luminescence properties in solution, however, often differ considerably from those on solid surfaces especially for silica gel stationary phases. This phenomenon is usually attributed to catalytic decomposition and fluorescence quenching effects [3, 4]. By using modern commercially available high-performance thin-layer chromatographic (HPTLC) silica gel plates catalytic decomposition may generally be controlled [3]. However, fluorescence quenching effects are more difficult to prevent since their mechanisms are not yet completely understood. Atmospheric oxygen is a well-known fluorescence quenching agent, although its influence in general is small [5] and may be diminished by flushing

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nitrogen over the HPTLC plate [6–8]. As a general rule it is assumed that adsorption of the sample onto the sorbent may provide additional non-radiative pathways for the loss of fluorescence excitation energy. These pathways seem to be no longer available when the plate is sprayed with or dipped in a viscous solvent, since the adsorbed compound is transferred to a liquid state in which other fluorescence-enhancing mechanisms may also be of importance [3–5].

Another sample treatment leading to fluorescence enhancement involves the creation of organized media; e.g. by formation of inclusion complexes with cyclodextrins (CDs), allowing enhancement of the spectroscopic properties of the guest molecule [9].

Fluorescence enhancers successfully employed in thin-layer chromatography (TLC) include liquid paraffin [10–13], triethanolamine [11, 13–15], Triton X-100 [3, 16, 17], dodecane [3], Fomblin Y-Vac [3], several surfactants and CDs [18]. Improved sample detection was thus obtained for fluorescent analytes such as polycyclic aromatic hydrocarbons [3, 18] and dansylated amino-acids [18].

In the present work, the effect of three different enhancers, Triton X-100, liquid paraffin and CDs, has been evaluated on the sensitive HPTLC fluorodensitometric determination of thiols of biological and pharmacological interest [19], derivatized with the fluorobenzoxadiazole reagents SBD-F [20–22] and ABD-F [22, 23]. The influence of enhancer and sample concentration, application method, drying conditions and measuring time after plate dipping has been investigated.

Experimental

Chemicals

SBD-F and ABD-F were purchased from Wako Chemicals (Neuss, FRG). *N*-Acetylcysteine, coenzyme A, cysteamine (2-aminoethanethiol), cysteine hydrochloride, glutathione and homocysteine were obtained from Aldrich, Janssen and Sigma (Belgium) and from Merck (FRG). Captopril was kindly supplied by N. V. Squibb (Belgium). All compounds were 99% pure and were used without further purification. The other chemicals used were of analytical grade, obtained from Merck (FRG) and UCB (Belgium).

Disodium EDTA (2.0 mM) was included in all thiol and derivatizing solutions to prevent metal-catalysed thiol oxidation. Re-distilled deionized water was used.

The fluorescence enhancers Triton X-100 (isooctylphenoxypolyethoxyethanol), Triton X-405 (70%, v/v, solution in water), liquid paraffin and α-, β- and γ-CDs were obtained from Janssen (Belgium).

Derivatizing procedure [20, 23]

In a 5-ml glass derivatizing vial, 1.0 ml of SBD-F (1.0 mM in 0.1 M sodium borate buffer (pH 9.5) with 2 mM disodium EDTA was added to 1.0 ml of each thiol solution (25 μM in 0.1 M sodium borate buffer (pH 9.5) with 2 mM disodium EDTA). The vial was capped, vortex mixed, heated in a water-bath at 60°C for 1 h and cooled in ice-water. All thiol solutions were freshly prepared each day. Nitrogen flushing and sonication were used to remove dissolved oxygen. A 200-nl aliquot of each reaction mixture was then analysed by HPTLC with fluorescence scanning densitometry.

A reagent blank solution (without thiol) was prepared similarly.

The derivatizing procedure of thiols with ABD-F was performed similarly but with less drastic conditions (5 min at 50°C and at pH 8.0).

Materials, instruments and chromatography [19]

Pre-coated HPTLC silica gel 60 plates (10 × 10 cm) without fluorescence indicator (Merck, FRG) were used. The derivatized sample solutions were spotted in 200-nl volumes at 5.0 mm intervals and at 1.0 cm from the bottom and borders of the plate using a Nano-Applicator in combination with a Nanomat application system (Camag, Switzerland). The plates were developed for about 5.0 cm in saturated twin-through chambers (Camag, Switzerland) with isopropyl ether–methanol–water–acetic acid (9:8:2:1, v/v/v/v). A standard UV lamp (Camag Type 29000, Switzerland) was used at 366 nm for viewing the adsorbed chromatographed derivatives. *In situ* quantitative scannings were performed with a Zeiss PMQ 3 densitometer (Zeiss, FRG) equipped with micro-optics, in the reflectance mode at λ_{ex} = 365 nm (Hg lamp), using a cut-off filter of 460 nm (FL 46, Zeiss, FRG). The chromatograms were recorded with an Ankersmit A40 recorder (Kipp and Zonen, Holland).

Measurements of fluorescence in solution were made on an Aminco Bowman spectrophotofluorimeter (American Instrument Co., MD, USA), fitted with a 150-W xenon arc lamp and a magnetic arc stabilizer, grating excitation and emission monochromators and an X-Y recorder for scanning excitation and emission spectra. Polymer fluorescence standard samples (ISA Belgium Groupe Instruments) were used for checking fluctuations in lamp intensity.

Fluorescence enhancement of the thiol-derivatives was performed by dipping (Desaga (FRG) dipping chambers) the developed plates in solutions of Triton X-100 in toluene and of liquid paraffin in *n*-hexane, at different concentrations (10–70%, v/v) for about 3 s, followed by scanning of the derivatives at several intervals of time after the dipping process. Aqueous solutions of α-, β- and γ-CDs (5–40 mM) were also evaluated as dipping reagents; for these solutions an infrared lamp (Quartz et Silice, France) was needed for drying the plates after dipping.

All measuring variables were held constant during the measurements. The mean result of at least five experimental values was calculated. No significant broadening of the spots was observed.

Results and Discussion

General remarks

The combination of HPTLC with fluorodensitometric measurements represents a most useful analytical method for the rapid, selective and sensitive determination of numerous analytes. Thiol compounds of biological importance such as coenzyme A, glutathione or the amino-acids cysteine and homocysteine, and of pharmaceutical interest such as the mucolytic agent acetylcysteine, the potent antihypertensive drug captopril or the radioprotector cysteamine may thus be specifically and reliably determined after prior derivatization with the fluorigenic reagents SBD-F or ABD-F [19].

Adsorption on to silica layers may produce a decrease in fluorescence emission when compared with solution measurements; this effect may be compensated by impregnating the plate with a non-volatile fluorescence-enhancing reagent such as Triton X-100, liquid paraffin or a CD, dissolved in a suitable solvent. Several factors are involved in this enhancement process: the type of enhancer and its way of application; sample and enhancer concentrations; drying and measurement conditions; and the time between plate dipping and measurement.

Type of enhancer and enhancement conditions

Two types of fluorescence enhancers were used in this study, viscous reagents (Triton X-100 and paraffin) and CDs (α-, β- and γ-CDs).

Viscous enhancers. Plate dipping was generally preferred to plate spraying since viscous solutions are difficult to spray with common nebulizing devices and an even distribution of the enhancer on the plate is difficult to achieve.

Table 1 shows the enhancement ratios (between the peak heights measured before and after plate dipping) and enhanced detection limits obtained with the viscous solutions of 40% (v/v) Triton X-100 in toluene and 40% (v/v) liquid paraffin in hexane (measured 35 min after dipping, $n = 5$). As observed, the highest enhancement ratio obtained was 12.5 with 40% (v/v) Triton X-100 for SBD–acetylcysteine. Unfortunately, other thiol-derivatives did not provide such high ratios. The detection limits (signal-to-noise ratio >2) of these thiolic compounds can thus be proportionally improved; low picogram limits per spot can be achieved with the systems described. Repetitive dipping of the plate in the same enhancer solution, with measurements between dips, did not provide improved results.

Toluene was chosen rather than chloroform or water as a solvent for Triton X-100 as it provided the highest enhancement ratios. Triton X-405 (70%, v/v, solution in water; Janssen, Belgium), a more viscous analogue of Triton X-100, was also tried for fluorescence enhancement purposes; a solution in ethanol did not provide higher enhancement ratios under the same experimental conditions. Liquid paraffin was also chosen in preference to its homologues, white soft paraffin (Winthrop Lab., USA) and paraffin wax (15%, m/m, hard paraffin wax + 85%, m/m, white soft paraffin; Winthrop Lab., USA) for similar reasons. Trials using various mixtures of Triton X-100 and liquid paraffin in chloroform (the most suitable solvent for dissolving mixtures of both viscous enhancers) showed that mixtures were not more effective than a dipping solution containing one enhancer.

Blank "enhancement" values were obtained by dipping the developed plates in the respective solvents (toluene or hexane). No relevant variations in the fluorescence signals were obtained with respect to the initial non-dipped values since the enhancement ratios were near 1 in most cases. The plates appeared to be free of solvent 35 min after dipping, without requiring any drying process; oven-heating (followed by acclimatization in a desiccator) even decreased the enhancement ratios with respect to those obtained with non-heated plates.

Possible changes in the excitation or in the emission wavelength maxima of the derivatives adsorbed on the plates due to the enhancement process could not be determined with the densitometric scanner used for these experiments. Comparative solution measurements could not be performed under the same enhancing conditions because the enhancer solutions were immiscible with water.

Cyclodextrin enhancers. Other fluorescence enhancers are the CDs, which were also evaluated as dipping reagents for the determination of SBD- and ABD-thiol derivatives. From the three commercially available CDs (α-, β- and γ-CDs), differing in the number of α-1,4-glycopyranose rings (6, 7 and 8, respectively, for α-, β- and γ-CD), β-CD provided the highest enhancement ratios for the majority of the fluorobenzoxadiazole derivatives, the enhancement order being β-CD > γ-CD > α-CD. In fact, α-CD did not (or only very slightly) give fluorescence enhancement. Elimination of the solvent (water)

from the plate was required to obtain enhancement response since measurement of wet plates resulted in lower fluorescence readings than those before dipping. Oven and infrared (IR) lamp-exposures (both at 55°C for 5 min after plate dipping) were compared to optimize the enhancement method. The IR plate heating method gave much better results than did oven-heating. Longer IR exposure times (>5 min) did not improve the results, the enhancement ratios actually decreasing with time. Drying the plate under the IR lamp at 55°C for 5 min after plate dipping followed by scanning 30 min later (plate acclimatized to room temperature in a desiccator) were the optimum conditions for CD enhancement. The enhancement ratios obtained with 20 μM aqueous β-CD dipping are shown in Table 1 together with the enhanced detection limits.

Measurements in solution carried out for comparative purposes showed a slight blue shift (±25 nm) of the emission maxima but not of the excitation maxima, of all thiol-derivatives when an equal volume of aqueous 20 μM β-CD was added to the derivatized thiol solution. As mentioned earlier, solid surface meaurements do not always follow the same luminescence behaviour as liquid measurements.

Figure 1 shows the chromatograms of SBD–cysteine measured before and after enhancement using several types of enhancers. The relative standard deviation of the complete fluorescence enhancement procedure was 3.50–4.10%.

Influence of sample concentration. For SBD–cysteine (chosen to represent fluoro-benzoxadiazole-thiol derivatives), a linear relationship was established between the peak height (fluorescence intensity, y) and concentration (x), over a range of 0–30 μM thiol: $y = 0.255x + 0.009$. After the fluorescence enhancement process the regression equations were: Triton X-100 (40%, v/v, in toluene), $y = 0.703x - 0.187$; liquid paraffin (40%, v/v, in hexane), $y = 0.604x + 0.038$; and β-CD (20 μM in water), $y = 0.255x + 0.009$.

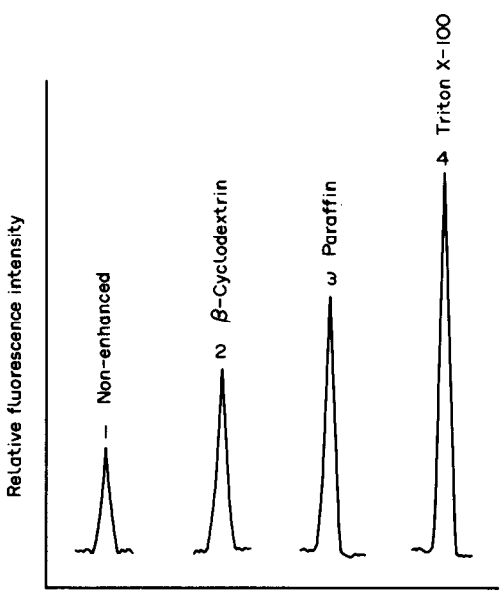

Figure 1
HPTLC–fluorodensitometry of 25 μM SBD–cysteine using different dipping enhancers: 1, non-enhanced; 2, 20 μM β-CD (ER = 1.69); 3, 40% (v/v) paraffin in hexane (ER = 2.68); 4, 40% (v/v) Triton X-100 in toluene (ER = 3.88) (Enhancement ratio (ER) measurements performed 35 min after plate dipping).

Table 1
Enhancement ratios (ER)* and enhanced detection limits (EDL)† of several thiol-derivatives with various fluorescence enhancers ($n = 5$)

	40% (v/v) liquid paraffin in hexane				40% (v/v) Triton X-100 in toluene				20 μM aqueous β-CD			
	SBD-derivative		ABD-derivative		SBD-derivative		ABD-derivative		SBD-derivative		ABD-derivative	
Thiol	ER	EDL	ER	EDL	ER	EDL	ER	EDL	ER	EDL	ER	EDL
Acetylcysteine	3.75	5.70	5.71	5.34	12.50	1.70	1.35	22.60	3.92	3.10	1.14	26.70
Captopril	2.76	6.60	2.10	8.40	2.00	9.10	1.06	17.10	1.80	10.10	1.00	18.10
Coenzyme A	2.06	14.50	3.23	9.50	1.93	15.50	1.33	23.10	1.18	25.30	1.14	26.90
Cysteamine	2.80	2.20	3.38	5.80	1.52	4.10	2.67	7.30	1.29	4.80	1.24	15.83
Cysteine	2.68	5.40	5.10	3.70	3.88	3.70	1.27	15.10	1.69	8.60	1.00	19.10
Glutathione	2.98	4.70	2.24	5.21	2.82	5.10	1.43	8.10	1.11	12.70	1.07	10.90
Homocysteine	2.14	5.20	4.30	2.10	3.24	3.40	1.37	6.50	1.82	6.16	1.32	6.70

*ER = ratio between the peak height after enhancement, measured 35 min after plate dipping, and peak height before enhancement.
†EDL = values are in pg per spot. Signal-to-noise ratio >2.

According to the calculated enhancement ratios for the different thiol concentrations, fluorescence enhancement seems to depend on the amount of sample, the enhancer being present in an excess because of the dipping process. Nevertheless, the enhancement ratios were constant for a given enhancer and sample for specific concentrations and conditions.

Influence of enhancer concentration. Different concentrations of enhancers (Triton X-100, liquid paraffin and β-CD) were tried on all SBD- and ABD-thiol derivatives to study their influence on fluorescence enhancement ratios. Significant increases in enhancement ratios were observed with increasing enhancer concentrations using the viscous reagents. Most of the derivatives presented their maximal enhancement ratios at reagent concentrations of 40–50%, v/v. Enhancer concentrations higher than 60%, v/v, were unsatisfactory owing to the limited solubility of the reagents in their respective solvents (toluene for Triton X-100 and hexane for liquid paraffin), producing uneven reagent dipping of the plate and poor reproducibility of the results.

For β-CD, a proportional relationship was observed between enhancer concentration and enhancement ratio. The highest enhancement ratios were observed with 20–40 μM β-CD concentrations, although these latter ratios were lower than those obtained with the viscous enhancers (Table 1). Concentrations higher than 40 μM of β-CD were not tried because of its limited solubility in water. It is interesting that in this case, the ABD-derivatives gave lower enhancement ratios (1–1.32) than did the SBD-derivatives (1.11–3.92).

Effect of the time between plate dipping and scanning on fluorescence enhancement. There appeared to be no significant influence of time (up to 2 h) after the dipping process on the observed enhancement ratios. For all enhancers, however, maximum enhancement ratios were observed about 35 min after plate dipping; these values decreased very slightly with time (Fig. 2).

Conclusions

The fluorescence enhancement of thiols derivatized with fluorobenzoxadiazole reagents by dipping the plates into enhancer solutions is valuable for increasing the

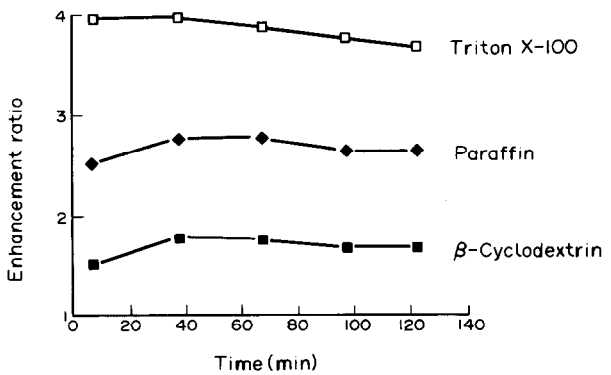

Figure 2
Influence of the time between plate dipping and scanning on the enhancement ratio of SBD–cysteine using the enhancers: ■, 20 μM aqueous β-CD; ◆, 40% (v/v) paraffin in hexane; □, 40% (v/v) Triton X-100 in toluene.

sensitivity of the assay of thiols. The enhancement process is essentially rapid and the fluorescence enhancement ratios do not appear to be significantly dependent on the time between dipping and scanning. Increase of the sample or enhancer concentration improves the detection limits, provided that the solubility of the enhancer permits such increase.

The fluorescence enhancement processes are recommended for application to other thiol compounds.

Other potential fluorescence enhancers, whether viscous (such as soft paraffin) or surfactants (such as sodium dodecyl sulphate, cetrimide or benzalkonium chloride) are worthy of consideration for possible use as fluorescence enhancers on solid supports.

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