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# Determination of thiols of biological and pharmacological interest by high-performance thin-layer chromatography and fluorescence scanning densitometry*

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**Abstract**: The application of high-performance thin-layer chromatography (HPTLC) with fluorescence scanning densitometry provides a simple, rapid and reliable system for the qualitative and quantitative determination of several thiols of biological and pharmacological interest. The determination of a mixture of thiols (captopril, coenzyme A, cysteamine, cysteine and glutathione), together with their disulphides may readily be performed by pre-chromatographic derivatization with the thiol-specific fluorobenzoxadiazole reagents SBD-F and ABD-F, followed by HPTLC separation on silica gel plates using isopropyl ether–methanol–water–acetic acid (9:8:2:1, v/v/v/v) as the developing solvent, and fluorodensitometric measurement of the fluorescing derivatives.

Detection limits of about 30 pg (coenzyme A) to 6 pg (cysteamine) per spot were achieved; the relative standard deviation (RSD) of the complete procedure was 1.16–3.2%.

**Keywords**: *Fluorescence detection; fluorobenzoxadiazoles; HPTLC; thiols.*

## Introduction

Selective, sensitive and reliable analytical methods for the determination of drugs and biological compounds are of major importance in pharmaceutical and biomedical analysis. Relevant thiol compounds, either biological, such as coenzyme A and glutathione, or pharmaceutically important such as the mucolytic agent acetylcysteine and the potent antihypertensive drug captopril, require highly specific and accurate analytical detection systems since the complex reactivity of the thiol function often causes serious problems for their reliable determination.

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Fluorescence labelling or chemical derivatization reactions yielding strongly fluorescing molecules seem to be most suitable for thiol trace analysis [1]. By coupling the simple, rapid and versatile analytical separation method of high-performance thin-layer chromatography (HPTLC) with fluorodensitometric measurements, an extremely sensitive and selective detection system is achieved [2–4]. The main advantages of this system, in comparison with the more widely used high-performance liquid chromatography (HPLC) are mostly derived from the former being an open-bed system, thus allowing simultaneous analysis of multiple samples under the same chromatographic conditions as well as repetitive scans of the same sample. Other advantages include simplicity, speed and excellent reliability due to the modern high quality stationary phases; use of disposable single-use plates avoids column maintenance or conditioning and loss of resolution due to irremovable samples [5].

Several thiol-specific fluorigenic reagents are available [6–8]. All show promise for the determination of thiols in various samples. Among these reagents the fluorobenzoxadiazole reagents SBD-F (ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate) and ABD-F (7-fluoro-4-sulphamoyl-2,1,3-benzoxadiazole) (Fig. 1) [9] present optimum fluorigenic and physicochemical features including lack of native fluorescence and low blank fluorescence, high reactivity towards thiols and excellent solubility and stability of reagents and of thiol derivatives [10]. Synthesized by Imai *et al.* [10, 11], these reagents have successfully been applied to the pre-column labelling of thiols in HPLC; in addition, the various factors that affect their fluorescence properties have been studied [11–19].

The present study describes the alternative use of the simple, rapid, reliable and sensitive method of HPTLC with fluorescence detection for the determination of SBD- and ABD-derivatives of thiols of biological and pharmacological interest, with low picogram detection limits.

## Experimental

### *Chemicals*

Re-distilled deionized water was used. The fluorigenic reagents SBD-F and ABD-F were purchased from Dojin (Tokyo, Japan) and from Wako Chemicals (Neuss, FRG). The thiol compounds *N*-acetylcysteine; coenzyme A; cysteamine (2-aminoethanethiol); cysteine hydrochloride; cysteine; cysteine ethyl ester hydrochloride; dimercaprol (BAL); dithioerythrithol; dithiothreithol; glutathione, reduced and oxidized; homocysteine;

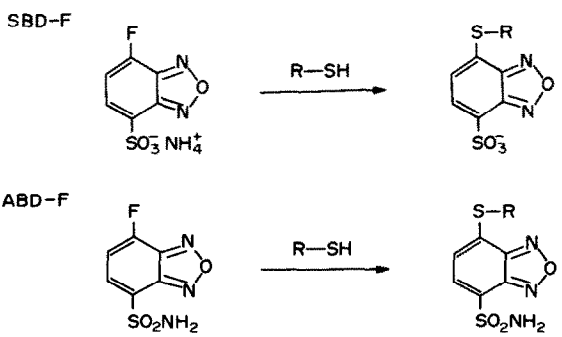


**Figure 1**
Chemical structures of SBD-F and ABD-F and their reaction with thiols.

homocystine; 2-mercaptoethanol; thioglycolic acid and thiolactic acid (2-mercaptopropanoic acid) were obtained from Aldrich, Janssen and Sigma (Belgium) and from Merck (FRG). Captopril was a gift from N. V. Squibb (Belgium). These chemicals were all 99% pure and were used without further purification. All other chemicals were of analytical grade (Merck, FRG; and UCB, Belgium).

Disodium EDTA (2.0 mM) was used in all thiol and reagent solutions to prevent metal-catalysed thiol oxidation.

*Derivatizing procedure* [10, 14]

To a 5-ml glass derivatizing vial containing 1.0 ml of SBD-F (1.0 mM) in 0.1 M sodium borate buffer (pH 9.5) with 2.0 mM disodium EDTA was added 1.0 ml of each thiol solution (25 μM in 0.1 M sodium borate buffer, pH 9.5, with 2.0 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*

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 vol 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-trough 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 developed spots. *In situ* quantitative scannings were performed with a Zeiss PMQ 3 densitometer (Zeiss, FRG) equipped with micro-optics, in the reflectance mode at $\lambda_{ex}$ = 365 nm (Hg lamp), and using a cut-off filter at 460 nm (FL 46, Zeiss, FRG). The chromatograms were recorded with an Ankersmit A40 recorder (Kipp and Zonen, Holland).

All HPTLC measurements reported in this study represent mean results of at least five experimental values.

*Simultaneous separation of thiols and disulphides* [19]

A 1.0-ml aliquot of a solution containing thiols and disulphides: reduced glutathione–oxidized glutathione, cysteine–cystine and homocysteine–homocystine (100 μM in 0.1 M sodium borate buffer, pH 9.3, with 2 mM disodium EDTA) was added to 1.0 ml of the derivatizing solution (1.0 mM ABD-F in 0.1 M sodium borate buffer, pH 9.3, with 2 mM disodium EDTA). The reaction mixture was heated at 60°C for 5 min. Ethyl acetate (4.0 ml) was then added to the reaction mixture to extract the excess of non-reacted ABD-F. The solution was vigorously shaken for 1 min and centrifuged for 5 min at 3000 rpm. A 0.4-ml aliquot of the aqueous layer was treated with 0.55 ml of SBD-F (1.0 mM in 0.1 M borate buffer, pH 9.3, with 2 mM disodium EDTA) and with 50 μl of a 10% (v/v) solution of tributylphosphine in dimethylacetamide; the reaction mixture was heated at 60°C for 20 min. The solutions were then analysed by chromatography.

## Results and Discussion

*Optimization of HPTLC conditions*

In order to select the most suitable mobile phase for the HPTLC determination of the thiol derivatives, different types of eluents (acid, basic and neutral) were tested. The most compact and intensely fluorescent spots at $R_f$ values of 0.15–0.93 for the thiol derivatives of major relevance were obtained with the following developing solvent: isopropyl ether–methanol–water–acetic acid (9:8:2:1, v/v/v/v). The excess of hydrolysed reagent was likewise examined; conveniently, this had migrated to the solvent front. The use of other stationary phases such as reversed-phase C18 or cellulose plates (all 10 × 10 cm, without fluorescence indicator; Merck, FRG) applied under the same chromatographic conditions did not give valuable analytical systems since diffuse-shaped spots were obtained that were situated either near the solvent front (RP-18 plates) or near the origin (cellulose plates).

The influence of eluent pH, acetic acid and water content on the chromatographic process was also investigated. Figure 2(a) shows the influence of eluent pH on the $R_f$ values of four representative thiol-SBD and -ABD derivatives. For this purpose, the acetic acid–water part (15%, v/v) of the optimum eluent was replaced by a 0.2 M acetic acid–0.2 M sodium acetate buffer at different pH values. Eluent pH values higher than 7 could not be tested owing to solvent immiscibility problems. A general increase in the $R_f$ values of the thiol derivatives was observed with increasing eluent pH values, with a consequent diffusion of the shape of spots. This phenomenon is attributed to the gradual neutralization of the derivatives, causing easier and faster elution of the less ionized compounds. For pH values above 4, the reagent spots at the solvent front interfere with the determination of compounds of high $R_f$ values, such as the SBD- and ABD-derivatives of acetylcysteine (about 0.88 and 0.90, respectively). The ABD-derivatives of cysteine and homocysteine, however, behave differently; their $R_f$ values generally decrease with increasing eluent pH.

Concordant results were obtained when investigating the influence of the acetic acid content of the eluent on the chromatographic behaviour of the assayed thiol derivatives.

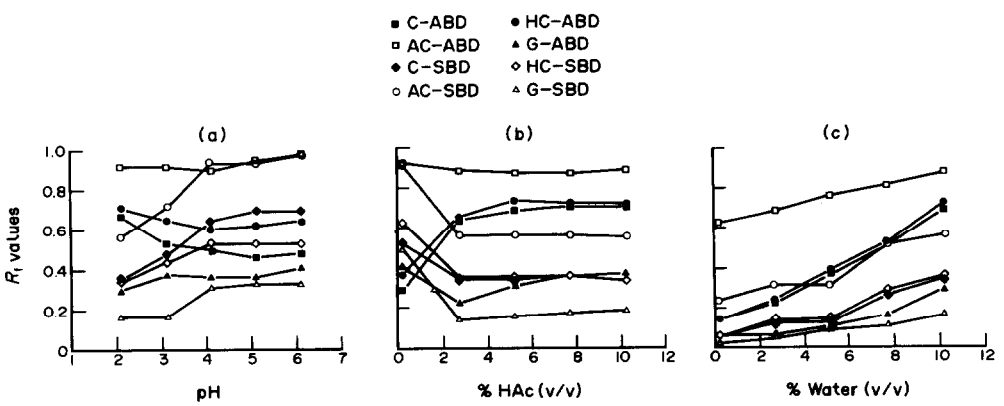


**Figure 2**
Influence of (a) eluent pH, (b) acetic acid content, and (c) water content on the $R_f$ values of: cysteine (C), homocysteine (HC), acetylcysteine (AC) and glutathione (G)-SBD and -ABD derivatives (see text for experimental conditions).

A slight addition (2.5%, v/v) of acetic acid to the eluent mixture of isopropyl ether–methanol–water (9:8:2, v/v/v) resulted in a decrease in the $R_f$ values (Fig. 2b). Thus more compact and clearly fluorescent spots were obtained. Higher proportions of acetic acid did not lead to considerable changes of the $R_f$ values obtained. However, similar to findings of the previous study, the ABD-derivatives of cysteine and homocysteine gave different results.

The influence of the water content of the eluent was also studied. Increasing amounts of water added to isopropyl ether–methanol–acetic acid (9:8:1, v/v/v) resulted in increasing $R_f$ values (Fig. 2c). Inclusion of water into the mobile phase is required to achieve migration of the polar derivatives in this straight-phase system. A 10% (v/v) concentration of water in the eluent appears to be adequate for the HPTLC analysis of the fluorescent derivatives; use of higher water concentrations may cause incompatibility problems with silica layers and increase in the development times (>35 min).

*Qualitative and quantitative determination of thiols*

Table 1 shows the different $R_f$ values of the SBD- and ABD-derivatives of the thiols under investigation.

The visual detection limits of the different SBD- and ABD-thiol derivatives excited at 366 nm are near 0.2 pmol (20–400 pg per spot). These limits can be lowered by applying luminescence-enhancing treatments of the layer, usually by spraying or dipping the plate using, for example, surfactants (such as Triton X-100 or sodium dodecyl sulphate), paraffins or cyclodextrin solutions [4, 20–23].

Based on the different $R_f$ values of the derivatives, the separation and determination of a mixture of the thiols can be achieved by following the proposed derivatizing and chromatographic procedure. The detection limits (signal-to-noise ratio >2) of the thiol derivatives of major importance are shown in Table 2. The best detection limit of 6.3 pg per spot was achieved for cysteamine–SBD. Calibration curves were linear in the picogram range, with a mean correlation coefficient of 0.9936. The RSD of the complete chromatographic procedure and densitometric measurement was 1.16–3.2%. The adsorbed fluorescent derivatives were highly stable when kept in a desiccator in the

**Table 1**
Mean $R_f$ values ($n$ = 5) of the SBD- and ABD-derivatives of several thiol compounds (see text for experimental conditions)

| Thiol | $R_f$ values SBD-derivative | ABD-derivative |
|---|---|---|
| Acetylcysteine | 0.58 | 0.90 |
| Captopril | 0.75 | 0.93 |
| Coenzyme A | 0.04 | 0.06 |
| Cysteamine | 0.49 | 0.70 |
| Cysteine | 0.31 | 0.60 |
| Cysteine ethyl ester | 0.45 | 0.91 |
| Dimercaprol | 1.00 | 1.00 |
| Dithioerythrithol | 0.84 | 1.00 |
| Dithiothreithol | 0.82 | 1.00 |
| Glutathione | 0.15 | 0.24 |
| Homocysteine | 0.33 | 0.70 |
| 2-Mercaptoethanol | 0.88 | 0.96 |
| Thioglycolic acid | 0.71 | 0.94 |
| Thiolactic acid | 1.00 | 1.00 |

**Table 2**
Mean detection limits ($n = 5$) of some SBD- and ABD-derivatives of relevant thiol compounds (see text for experimental conditions)

| Thiol | Detection limits* (pg per spot) SBD-derivative | ABD-derivative |
|---|---|---|
| Acetylcysteine | 21.5 | 30.5 |
| Captopril | 18.3 | 18.0 |
| Coenzyme A | 29.9 | 30.7 |
| Cysteamine | 6.3 | 19.6 |
| Cysteine | 14.7 | 19.1 |
| Glutathione | 14.1 | 11.7 |
| Homocysteine | 11.2 | 8.9 |

* Signal-to-noise ratio >2.

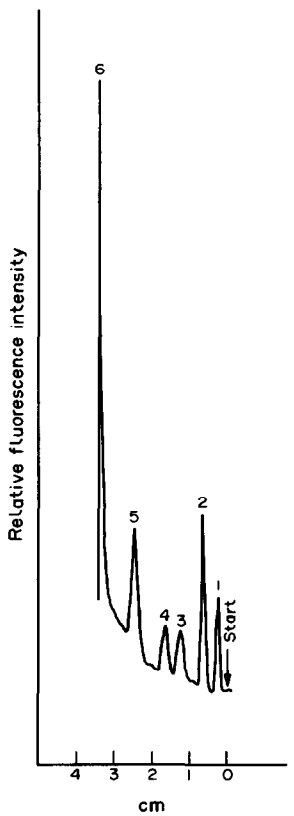


**Figure 3**
Chromatogram of a mixture of SBD-thiol derivatives: 1, coenzyme A; 2, glutathione; 3, cysteine; 4, cysteamine and 5, captopril. 6, Solvent front. Each thiol at 25 μM (see text for experimental conditions).

absence of light. Unaltered fluorescence responses were obtained even 24 h after development when stored under dry and dark (aluminium foil wrapped) conditions. Figure 3 shows the HPTLC separation of five thiols of biological and pharmacological interest after pre-plate SBD-F derivatization and fluorescence scanning densitometry. The fluorescence background increases across the plate owing to the hydrolysis of the excess of reagent.

*Simultaneous separation and identification of thiols and disulphides*

The procedure has also been applied to the simultaneous determination of thiols and disulphides, following the derivatizing procedure proposed by Toyo'oka *et al.* [19].

The free thiols are first derivatized with ABD-F, the excess of reagent being eliminated by extraction with ethyl acetate. Then, after reduction to their free thiols with tributylphosphine, a second derivatization process of the disulphides is performed with SBD-F. HPTLC separation with fluorescence detection of the derivatives is then applied as previously described. Separations of oxidized and reduced glutathione, of cystine and cysteine and of homocystine and homocysteine were performed accordingly. Figure 4 shows the fluorodensitometric separation of reduced glutathione (as ABD-derivative, peak 2) and oxidized glutathione (as SBD-derivative after reduction to the free thiol, peak 1); a clear separation of both derivatives was achieved. Quantitative determinations and application to biological samples is presently the subject of research.

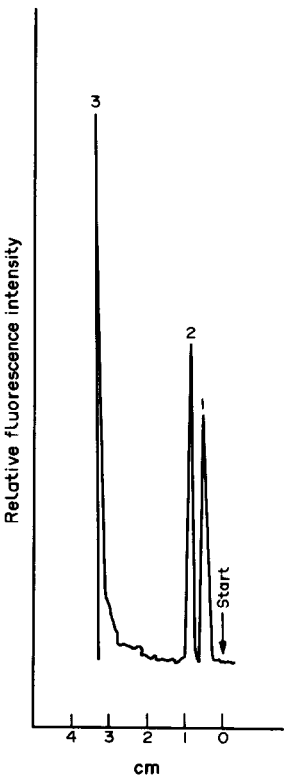


**Figure 4**
Chromatogram of 1, oxidized glutathione (reduced with tributylphosphine) -SBD; and 2, reduced glutathione -ABD derivatives, each at 35 μM. 3, Solvent front (see text for experimental conditions).

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