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Note

Application of high-performance thin-layer chromatography–fluorescence densitometry to the simultaneous determination of reduced and oxidized glutathione

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The tripeptide glutathione (GSH), γ-glutamylcysteinylglycine, is the major non-proteic thiolic compound of living plant and animal cells. Its important role in cellular metabolism includes the detoxification of xenobiotics, regulation of enzyme activity by disulphide interchange, protection against cell damage by ionizing radiation or by active oxygen species and transport of amino acids[1,2]. It is mainly the reduced form of glutathione that is present in tissues. However, the simultaneous determination of reduced (GSH) and oxidized (GSSG) glutathione could be useful for observing possible disorders in glutathione metabolism[3].

There are several analytical methods available for the determination of thiols and disulphides. Enzymatic, chromogenic and fluorogenic reactions as well as electrochemical detection systems preceded by liquid chromatography have been widely reported[4–6]. However, these assays often present serious drawbacks. Enzymatic reactions are mostly specific for a particular thiol or disulphide, whereas in electrochemical systems the selectivity, stability and reproducibility of the electrode may sometimes cause problems.

Based on the high-performance liquid chromatographic system recently described by Toyo'oka *et al.*[6], this paper proposes an alternative high-performance thin-layer chromatographic (HPTLC) system for the simultaneous determination of GSH and GSSG. In combination with fluorescence scanning densitometry and prior fluorescent labelling of the thiol group, a relatively simple, fast and versatile separation method is coupled to a sensitive and selective detection system, avoiding several inconveniences related to column liquid chromatography[7].

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EXPERIMENTAL

Chemicals

The thiol-specific fluorogenic reagents 7-fluoro-4-sulphamoyl-2,1,3-benzoxadiazole (ABD-F) and ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F)[8] (Fig. 1) were purchased from Wako (Neuss, F.R.G.). GSH was obtained from Merck (Darmstadt, F.R.G.) and GSSG from Aldrich (Brussels, Belgium). The reducing agent tributylphosphine (TBP) was dissolved in dimethylacetamide (DMA), both from Janssen Chimica (Beerse, Belgium). Redistilled, deionized water was used throughout. All other chemicals were of analytical-reagent grade and used without further purification.

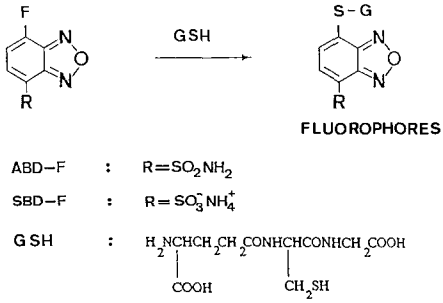

Fig. 1. Structures of the fluorogenic reagents ABD-F and SBD-F and reaction with glutathione.

Apparatus

Derivatization reaction. A standard vortex reagent mixer, centrifuge and temperature-controlled water-bath were used.

Chromatography. The derivatized samples were spotted on pre-coated silica gel 60 HPTLC plates (10 × 10 cm) without fluorescence indicator (Merck) using a Nano-Applicator in combination with a Nanomat application system (Camag, Muttenz, Switzerland). Saturated twin-trough chambers (Camag) were used to develop the plates. A standard UV lamp (Camag Type 29 000) was used at 366 nm for viewing the adsorbed fluorescent derivatives.

Fluorodensitometry. In situ quantitative scannings were performed with a PMQ 3 densitometer (Zeiss, Oberkochen/Württemberg, F.R.G.) equipped with micro-optics, in the reflectance mode, using a mercury lamp source at a $\lambda_{exc.} = 365$ nm and an emission cut-off filter for $\lambda_{em.} > 460$ nm (FL 46; Zeiss). The chromatograms were registered with an Ankersmit A40 recorder (Kipp and Zonen, Delft, The Netherlands) installed at the appropriate amplifying voltages. Integration of the chromatograms was done with a Chromatopac C-R3A integrator computer (Shimadzu, Kyoto, Japan). All HPTLC results represent average values of at least ten measurements.

Procedure

Derivatization reaction[6]. To an aliquot of 1.0 ml of a mixed solution containing GSH and GSSG (each at a working concentration of 100 μM) in 0.1 M sodium borate buffer (pH 9.3, containing 2 mM disodium EDTA) was added an equal volume of

ABD-F (1.0 m*M*) in 0.1 *M* sodium borate buffer (pH 9.3, 2 m*M* disodium EDTA). The reaction mixture was vortex-mixed, heated in a water-bath at 60°C for 5 min and cooled in ice. Ethyl acetate (4.0 ml) was then added to the mixture to extract the excess of unreacted ABD-F. The solution was shaken vigorously for 1 min and centrifuged at 1500 *g* for 5 min. A 400-μl volume of the lower aqueous layer was then treated with 550 μl of SBD-F [1.0 m*M* in 0.1 *M* sodium borate buffer (pH 9.3, 2 m*M* disodium EDTA)] and with 50 μl of the reducing agent [10% (v/v) TBP in DMA], followed by heating of the reaction mixture at 60°C for 20 min. An HPTLC analysis was then carried out.

Chromatography[7]. The derivatized solutions, acclimatized to room temperature, were spotted in 200-nl volumes at 5.0-mm intervals and at 1.0 cm from the bottom and borders of an HPTLC silica gel 60 plate. The plates were then developed in saturated twin-trough chambers for about 5.0 cm using diisopropyl ether–methanol–water–acetic acid (45:40:10:5, v/v) as an optimized eluent. The separated ABD and SBD derivatives were subsequently measured by fluorescence scanning densitometry.

RESULTS AND DISCUSSION

Derivatization conditions[6]

Disodium EDTA was added to all sample solutions in order to prevent metal-catalysed thiol oxidation. The thiol-specific fluorogenic reagents ABD-F and SBD-F bearing the fluorobenzoxadiazole structure (Fig. 1) were chosen owing to their high reactivity and selectivity towards thiols, and also because of the optimum stability and luminescence properties of their thiol derivatives[8]. ABD-F was used as the first derivatizing reagent because, in comparison with its homologue SBD-F, the former's reduced water solubility allows easier removal of the unreacted excess of ABD-F reagent (extraction with ethyl acetate). The optimum reaction conditions (solvents, pH, temperature and time of reaction, extraction procedure, reducing agent, etc.) recommended by Toyo'oka *et al.*[6] for the derivatization of thiols and disulphides prior to chromatography were adopted in this work.

Optimization of HPTLC conditions

Different types of eluents (acidic, neutral and alkaline) were tried as developing solvents for the separation of the glutathione ABD and SBD derivatives. The most compact and intensely fluorescent spots for the HPTLC silica gel system formerly described were obtained with the solvent mixture diisopropyl ether–methanol–water–acetic acid (45:40:10:5, v/v), providing R_F values of 0.24 for GSH (corresponding to GSH–ABD) and at 0.15 for GSSG (corresponding to the SBD derivative after TBP reduction of GSSG to the free thiol). The use of other stationary phases such as reversed-phase C_{18} or cellulose HPTLC plates provided, under the same experimental conditions, highly diffuse spots that could not be quantitated, situated at $R_F > 0.90$ (RP-18) and < 0.10 (cellulose).

The influence of increasing proportions of acetic acid and water in the eluent[7] on the R_F values of the ABD and SBD derivatives is shown in Fig. 2. As observed, the addition of acetic acid to the eluent drastically decreases the R_F values of both derivatives, providing more compact and highly fluorescent spots. Apparently, protonation of the sulphonic acid function makes the SBD derivatives migrate at lower

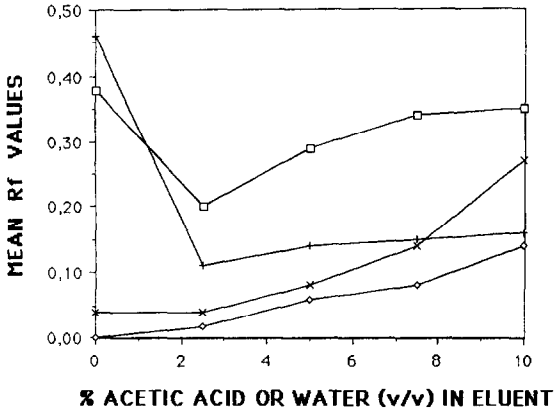

Fig. 2. Influence of acetic acid and water concentrations on R_F values of GSH derivatives (n = 10): □, G-ABD (acetic acid); +, G-SBD (acetic acid); ×, G-ABD (water); ◇, G-SBD (water). G-ABD = GSH derivatized with ABD-F; G-SBD = GSSG reduced with TBP and derivatized with SBD-F.

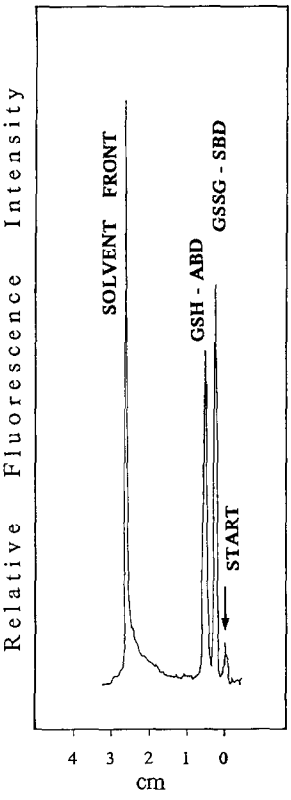

Fig. 3. HPTLC of reduced (GSH-ABD) and oxidized (GSSG-SBD) glutathione (each at a working concentration of 100 μM) following the described method.

R_F values. Similar results were obtained when the influence of eluent pH was studied. Owing to salt formation, a general increase in R_F values was observed with increasing eluent pH, accompanied by diffusion of the spots. Eluent pH values higher than 7 could not be tested owing to solvent inmiscibility problems. The optimum acetic acid content was of 5% (v/v) with a final pH of 2. With respect to the hydrophilicity of the eluent, an increase in the R_F values is clearly observed with increasing water concentration, a 10% (v/v) concentration providing satisfactory separations. Higher water concentrations in the eluent are not recommended as they are not compatible with the silica layer and as they tend to increase the developing times.

Determination of reduced and oxidized glutathione

Fig. 3 shows the chromatogram obtained with the described HPTLC system. The deviation of the baseline towards the solvent front is due to the migration of the hydrolysis product of the excess of fluorogenic reagents.

The detection limits (signal-to-noise ratio $\geqslant 2$) were 18 and 63 pg per spot for GSH and GSSG, respectively, although these values might be lowered by applying a luminescence enhancing treatment[9–13].

Following the proposed method, linear calibration graphs for GSH ($y = -2.536 + 0.268x, r = 0.99$) and GSSG ($y = 0.757 + 0.286$x, $r = 0.99$) were obtained for plots of relative fluorescence intensity (peak height, y) of the derivatives *versus* the working concentration of GSH or GSSG (x) over the range 0–100 μM. The reproducibility of the overall derivatizing and chromatographic procedure is of 4.3% ($n = 10$).

The proposed method for the determination of GSH and GSSG is relatively simple, rapid and selective and may be applied to the determination of other thiols and disulphides. The use of an internal standard is recommended for the accurate determination of low levels of analytes in biological samples, and this aspect is currently being investigated.

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