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Micro liquid chromatography with fluorescence detection of thiols and disulphides

B. LIN LING[a,*]

Department of Pharmaceutical Chemistry and Drug Quality Control, Pharmaceutical Institute, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)

C. DEWAELE

Bio-Rad RSL, Begoniastraat 5, 9810 Nazareth (Belgium)

and

W. R. G. BAEYENS

Department of Pharmaceutical Chemistry and Drug Quality Control, Pharmaceutical Institute, State University of Ghent, Harelbekestraat 72, B-9000 Ghent (Belgium)

ABSTRACT

Several biological thiols (cysteine, homocysteine and glutathione) together with their disulphides (cystine, homocystine and oxidized glutathione) could be simultaneously determined by micro liquid chromatography (LC) with fluorescence detection. Based on an existing procedure, the samples were first treated with the thiol-specific fluorigenic reagent ABD-F followed by disulphide reduction with tributylphosphine and SBD-F derivatization of the reduced disulphides. Different mobile phases, columns, injection volumes and detection systems were tried for the optimization of the separation. Quantitative studies gave detection limits at the picogram level per 60-nl injection volumes. Calibration graphs were linear with correlation coefficients >0.999. The possibility of applying gradient elution to this system represents an advantage over high-performance thin-layer chromatographic assays. Likewise, the advantages of micro-LC over classical high-performance liquid chromatography may contribute to the choice of the most suitable system depending on the analytical requirements.

INTRODUCTION

The identification and determination of biological thiols and disulphides in living organisms is of considerable importance for assessing the normal functioning of cell metabolism. Certain biochemical disorders may be detected by observing the altered thiol-to-disulphide ratio. Amongst these are cystinuria, homocystinuria and haemolytic anaemia due to glutathione synthetase deficiency [1,2].

Numerous methods for the selective and sensitive determination of the highly reactive thiol group have been previously described. Most are based on high-performance liquid chromatography (HPLC) with UV absorption, electrochemical or fluo-

[a] On leave from the Department of Bromatology and Pharmaceutical Analytical Techniques, Faculty of Pharmacy, Complutense University of Madrid, Ciudad Universitaria, E-28040 Madrid, Spain.

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rescence detection [3–5]. However, there have been fewer studies on the simultaneous determination of oxidized and reduced thiols. Traditionally, these assays were performed by reducing the disulphides to the corresponding thiols with a suitable reducing agent prior to their detection. The concentration of disulphide was then calculated by subtraction from the total thiol concentration (thiol and disulphide) [6,7]. Other methods include the determination of GSH and cysteine together with their disulphides by HPLC and dual electrochemical detection [8,9]. However, these studies did not consider the specific behaviour of homocysteine, the essential biological amino acid, a homologue of cysteine. Toyo'oka *et al.* [10] reported on a simultaneous determination method for thiols and disulphides based on their selective derivatization with two fluorogenic labelling reagents, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F) and 4-(aminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) (Fig. 1). They described the optimum reaction conditions and the main physicochemical properties of the reagents and the derivatives [11]. The method was then succesfully applied to the analysis of rat and hamster tissues [12].

This paper is based on the selective thiol and disulphide derivatization procedure optimized by Toyo'oka *et al.* [10], applied to the recently introduced and rapidly growing field of micro-LC. Amongst the main advantages of this technique over conventional HPLC are the low consumption of mobile phase, stationary phase and sample, apart from the better permeability and efficiency of the columns [13]. As described in a previous paper [14], conventional HPLC equipment could be adapted to micro-LC analysis by using a split flow system in combination with a laboratory-made square quartz cell for fluorescence measurements. In this work, the latter system was optimized further for the simultaneous determination of thiols together with their disulphides.

EXPERIMENTAL

Chemicals

Glutathione (GSH) and cystine were obtained from Merck (Darmstadt, Germany), cysteine and homocystine from Sigma (St. Louis, MO, U.S.A.) and homocysteine and oxidized glutathione (GSSG) from Aldrich (Beerse, Belgium). All compounds were chemically pure and used as received.

The fluorigenic derivatizing reagents SBD-F and ABD-F were obtained from Wako (Neuss, Germany). Tributylphosphine (TBP), used for reducing the disulphides, was dissolved in dimethylacetamide (DMA), both from Janssen (Beerse, Bel-

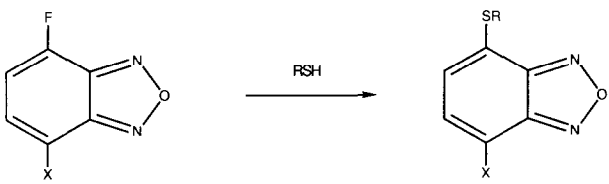

Fig. 1. Structures of ABD-F and SBD-F and general reaction with thiols (RSH).

gium). Acetonitrile and water for the mobile phase were of HPLC grade (Alltech, Deerfield, IL, U.S.A.). Disodium EDTA (Merck) was added to all thiol and reagent solutions at a concentration of 2.0 m*M* to prevent metal-catalysed thiol oxidation.

Derivatization reaction

The present sample preparation method was initially proposed by Toyo'oka *et al.* [10]. Standard thiol and disulphide solutions were prepared by mixing equal volumes of each thiol (cysteine, homocysteine and GSH) and each disulphide solution (cystine, homocystine and GSSG), all compounds dissolved in 0.1 *M* aqueous sodium borate buffer (pH 9.5) containing 2.0 m*M* disodium EDTA. To a 1-ml aliquot of this mixture was then added an equal volume of the first fluorogenic reagent, ABD-F (1.0 m*M* dissolved in the above-mentioned borate buffer solution). The reaction mixture was vortex mixed, heated in a water-bath at 60°C for 5 min and cooled in ice. The excess of unreacted ABD-F was extracted by the addition of 4.0 ml of ethyl acetate, followed by vigorously shaking for 1 min and centrifuging at 1850 *g* for 5 min. To 400 μl of the lower aqueous layer were added 550 μl of SBD-F (1.0 m*M* dissolved in the previously mentioned 0.1 *M* sodium borate buffer solution) and 50 μl of the reducing agent TBP, 10% (v/v) in DMA. This final reaction mixture was heated at 60°C for 20 min, cooled in ice and subjected to micro-LC analysis with fluorescence detection at room temperature.

Apparatus and chromatography

Conventional HPLC equipment was adapted to micro-LC studies by connecting the pump (Model 5560 gradient elution delivery system; Varian, Walnut Creek, CA, U.S.A.) to a T-flow-split system (Valco, Houston, TX, U.S.A.), which divides the mobile phase between the micro-LC column (250 × 0.32 mm I.D. fused-silica capillary filled with 5-μm RoSiL C_{18}; Bio-Rad RSL, Eke, Belgium) and a by-pass conventional HPLC column (5-μm RoSiL C_{18}, 150 × 4.6 mm I.D.). An internal volume injector (60–1000 nl, Valco CI4W) and a 10-μl Valco external injector loop (CV6U) was used for the micro-LC system. The optimized mobile phase conditions were gradient elution with 0.15 *M* H_3PO_4 (A)–acetonitrile (B) from 92:8 to 70:30 (v/v) in 15 min followed by isocratic elution with A–B (70:30, v/v) for 5 min. Chromatography was carried out at room temperature at a flow-rate of 3.0 μl/min, which was frequently checked by connecting an empty 10-μl syringe to the column end and timing the advance of the liquid meniscus. The chromatographed samples were detected by means of a fluorescence detector (Shimadzu Model RF-535; Pleuger, Wijnegem, Belgium) at λ_{exc} = 380 nm, λ_{em} = 510 nm, into which a laboratory-made 312-nl square quartz cell was inserted (0.25 × 0.25 × 5.0 mm). For comparative purposes, a Model 272 UV detector (Wescan, Santa Clara, CA, U.S.A.) containing a similar laboratory-made square quartz cell was used. For recording the signals, a Model 2020-000 recorder, (Linear Instruments, Reno, NV, U.S.A.) was employed. Integration was performed on a Chromatopac C-R3A integrator system (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

Optimization of the micro-LC chromatographic system

Mobile and stationary phases. Based on the chromatographic separation of a mixture of several SBD- and ABD-labelled thiol derivatives [14], the following gradient eluent was initially tried: 0.15 *M* H_3PO_4 (A)–acetonitrile (B), from 95:5 to 70:30 (v/v) in 15 min followed by isocratic elution with A–B (70:30, v/v) for 5 min at a flow-rate of 3.0 μl/min. This resulted in an optimum separation of the three ABD derivatives corresponding to the thiols cysteine, homocysteine and GSH. The disulphide compounds, which had initially been reduced to the corresponding thiols with TBP and derivatized with SBD-F, eluted later and overlapped under the above conditions. Therefore, less acetonitrile in the gradient composition was subsequently tested followed by a higher initial H_3PO_4 content in the eluent. The final optimized eluent conditions were a gradient of A–B from 92:8 to 70:30 (v/v) in 15 min followed by isocratic elution with A–B (70:30, v/v) for 5 min. The resulting chromatogram is shown in Fig. 2. The SBD derivatives eluted earlier than the ABD derivatives, although the order of elution was the same within one group of thiols (*i.e.*, cysteine first followed by homocysteine and GSH), probably owing to the increasing hydrophobic-

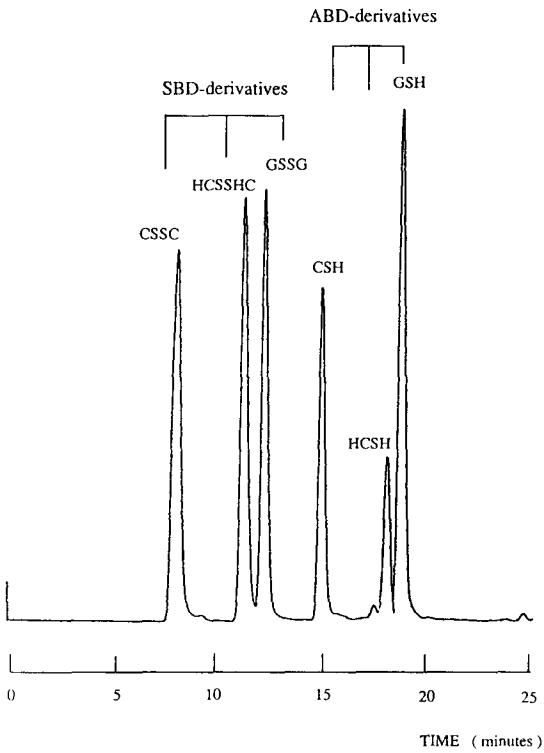

Fig. 2. Chromatogram obtained from micro-LC with fluorescence detection of 60 nl of a mixture containing CSH (cysteine) (125 μg/ml), HCSH (homocysteine) (13 μg/ml), GSH (glutathione) (45 μg/ml), CSSC (cystine) (180 μg/ml), HCSSHC (homocystine) (29 μg/ml) and GSSG (oxidized glutathione) (68 μg/ml).

ity and dimensions of the compounds. Even though the retention times of homocysteine and GSH were similar in both types of derivatives, a better separation of the four compounds in one run either by inclusion of less H_3PO_4 or by increasing the acetonitrile content was not feasible, as the SBD and the ABD derivatives overlapped, respectively. The eluent indicated above gave the most acceptable results.

Fused-silica capillaries (250 × 0.32 mm I.D.) packed with 5-μm C_{18} silica gel were used throughout. Smaller particle sizes did not provide significant changes in the chromatographic separation. The application of longer columns (1 m), described previously [14], resulted in better resolution but required longer analysis times.

Injection volume. Once the column and mobile phase had been optimized, different injection volumes were tried in an attempt to increase the sensivity without a significant decrease in efficiency. Injection volumes of 60, 200, 500, 1000 and 10000 nl were tried, giving in all instance an acceptable separation of the derivatives owing to the "on-column focusing" effect [15] and the use of gradient elution. This allows the assay of samples prepared for conventional HPLC on micro-LC systems. However, the first-eluting peak (cysteine-SBD) suffered considerably from high injection volumes as it was severely broadened and therefore decreased the overall resolution of the separation. Injection volumes of 60 nl were therefore preferred and used throughout.

Detection system. Fluorescence detection was preferred to UV absorption detection because of the higher sensitivity and selectivity. UV absorption measurements using a similar laboratory-made square quartz cell gave a considerable baseline drift owing to refractive index changes in the gradient elution system and also a noisy background signal. As much as a 10-fold increase in the detection limits was observed on going from fluorescence to UV measurements. "On-column" fluorescence detection [14] was tried previously but did not improve the results.

Qualitative and quantitative separation of thiols and disulphides

The chromatogram in Fig. 2 shows the optimized simultaneous separation of the three thiols cysteine, homocysteine and GSH as their ABD derivatives together with their respective disulphides cystine, homocystine and GSSG as their SBD deriv-

TABLE I

RETENTION TIMES AND DETECTION LIMITS OF THE VARIOUS THIOLS (DETECTED AS ABD DERIVATIVES) AND DISULPHIDES (DETECTED AS SBD DERIVATIVES)

Compound	Retention time (min)	Detection limit (pg per 60 nl injection)
Thiols		
Cysteine	16.3	83
Homocysteine	19.7	18
GSH	20.5	20
Disulphides		
Cystine	8.7	108
Homocystine	12.2	15
GSSG	13.3	35

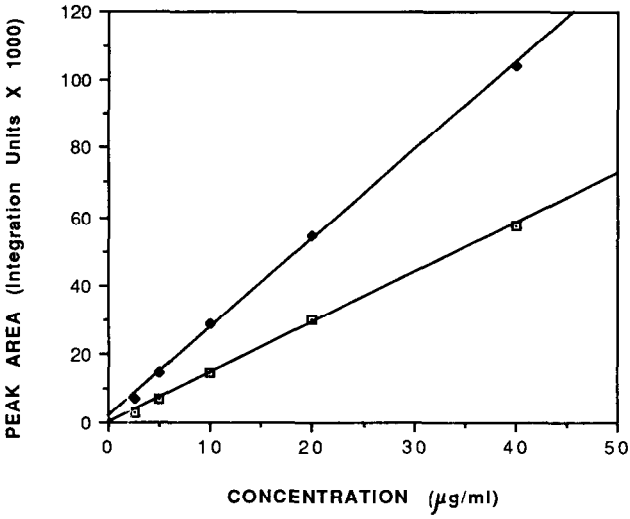

Fig. 3. Calibration graphs for (◆) GSH and (□) GSSG. GSH: $y = 1.8962 + 2.5702x$; $R^2 = 0.999$. GSSG: $y = -0.18450 + 1.4523x$; $R^2 = 0.999$.

atives. Their retention times and detection limits (signal-to-noise ratio >2) are given in Table I. The relative standard deviations were 0.690% and 0.810% for the retention time and peak areas, respectively (for $n = 10$, GSH-SBD). It may be concluded that, in general, the ABD derivatives gave higher fluorescence signals than the corresponding SBD derivatives (note that the signals of the SBD derivatives should correspond to more or less double the respective signals of the thiol derivatives). The relatively poor detection limit observed for cysteine-ABD is probably a result of its low stability under the conditions of measurement and the shifts of its luminescence parameters [10]. Likewise, the detection limit of cystine was poor, in addition to its limited solubility in borate buffer media, which made heating and stirring necessary for complete dissolution. Calibration graphs (fluorescence intensity signals *versus* thiol or disulphide concentration) for GSH and GSSG as representative thiol derivatives were linear in the range 0–40 μg/ml (Fig. 3), with correlation coefficients >0.999. Blank runs (containing no thiol compound) gave no peaks under the same chromatographic conditions.

Comparison with other chromatographic systems

In a previous paper [16] the applied derivatization procedure was similarly performed in high-performance thin-layer chromatographic experiments, and GSH and GSSG could be adequately separated and determined. However, cysteine, homocysteine and their disulphides could not be simultaneously separated as the R_F values of their derivatives were too close for reliable quantification.

The advantage of the present micro-LC system lies in its versatility. The possibility of applying gradient elution to the system allowed the selective separation of all the thiols and disulphides simultaneously in one run. In this instance the detection limits of GSH and GSSG were similar in both chromatographic systems.

In conventional HPLC [10], the detection limits achieved were slightly better for the derivatives assayed, although the previously mentioned advantages of micro-LC [13] may be of considerable relevance whenever a chromatographic system is to be chosen for a particular purpose.

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