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HPLC determination of biologically active thiols using pre-column derivatisation with 5,5′-dithio-(bis-2-nitrobenzoic acid)

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Abstract

5,5′-Dithio-(bis-2-nitrobenzoic acid), Ellmans reagent (ESSE), is used as a pre-column derivatisation reagent for the determination of biologically active thiols by HPLC. D-penicillamine, *N*-acetyl-D-penicillamine, *N*-acetylcysteine, cysteine, captopril and thiomalic acid all give well resolved derivatives. The calibration graph and reproducibility (%R.S.D. $\pm$ 1.3%) for the analysis of glutathione indicates that the method could be used for quantitative analysis. ESSE is widely used as a reagent in thiol determinations by electronic spectroscopy via the detection of the Ellmans anion (ES^-) generated without any prior separation procedures. However, there are considerable reservations over its use for the spectrophotometric determination of thiols because of the possibility of side reactions which generate another Ellmans based species (ESO_2^-). The assay described determines the thiol as a derivatised mixed disulphide (ESSR) and since speciation between the anion ES^- and the oxidation product ESO_2^- occurs it enables the process of oxidation to be monitored simultaneously. © 1997 Elsevier Science B.V.

Keywords: HPLC; Thiol assay; Analysis; Ellmans reagent; Pre-column derivatisation

1. Introduction

Compounds containing thiol groups are used in the treatment of a number of diseases. For example, captopril and more recently *N*-acetylcysteine are used to treat patients suffering from heart failure [1,2] and D-penicillamine is used in the treatment of rheumatoid arthritis [3]. One perceived action of thiols in vivo is as anti-oxidants [4] which protect cells and tissue by actively scavenging toxic oxygen species [5]. In addition, naturally occurring thiols such as glutathione and cysteine have important metabolic roles. All these molecules can react with oxidants released in the disease process (e.g. reperfusion shock) [6–8]. Thus, there is a need for qualitative and quantitative methods of analysis of mixtures of biologically active thiols and the products of their reaction in vivo.

The advantage of an HPLC procedure is that it enables speciation between closely related compounds. Previous HPLC methods for thiol analy-

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sis have used detection by electrochemistry [9,10], fluorimetry [11–13] and electronic spectroscopy [14,15] after pre- and post-column derivatisation. The electronic spectroscopic method employed 5,5′-dithio-dinicotinic acid (TNA) [16]. However, the similarity between the reactions of the thiols means that identification of each species is best achieved by chromatography coupled with a diode array detector. This generates molecularly specific spectra from separated peaks on the chromatogram giving a good definition of each species. In addition Ellmans reagent (ESSE), 5,5′-dithio-(bis-2-nitrobenzoic acid) is widely used for the analysis of sulphydryl groups in biological systems via the determination of the liberated anion (ES^-, Eq. (1)). However, the use of Ellmans reagent requires some care as a number of possible interfering reactions of the anion (ES^-) can occur leading to an oxidised product and therefore an error in the analysis. These products can be difficult to detect by electronic spectroscopy since they lead to only minor changes in the peak position of the electronic spectra of the products of reaction but by altering intensity they can act as interferants. Using HPLC separation coupled with diode array detection as an extension of the standard thiol assay using Ellmans reagent, speciation by chromatography could provide the separate detection of each species in the reactions and enable monitoring of unwanted oxidation products directly.

2. Experimental

2.1. Materials and methods

N-acetylcysteine was provided by Zambon Research (Milan, Italy), *N*-acetyl-penicillamine and penicillamine by Lilly Research (Windlesham, Surrey, UK). Sodium dihydrogen ortho-phosphate, sodium hydroxide and HPLC grade methanol (Fisons, Loughborough, Leicestershire, UK), 5,5′-dithio-(bis-2-nitrobenzoic acid), glutathione, cysteine, captopril and D-penicillamine (Sigma, Poole, Dorset, UK) and thiomalic acid (Aldrich, Gillingham, Dorset, UK) were used as received. Phosphate buffered saline (PBS) was prepared with sodium dihydrogen phosphate (0.125 M) and sodium chloride (0.154 M). The pH was adjusted to 7.4 with sodium hydroxide.

2.2. Apparatus

A Waters 600E HPLC system and Waters 990 photodiode array detector with a spectral range of 200–800 nm were used. Manual injections were carried out using a Waters U6K chromatography injector with a 75 μl sample loop. Separation were carried out at ambient temperature on a reverse phase hypersil octadecylsilane (ODS1) column (250 mm × 4.6 mm i.d.). All solvents used were degassed at 20 ml min^{-1} with helium and filtered before entering the column. Using a gradient elution programme where the mobile phases were 100% PBS and PBS–methanol (82.5:17.5%, v/v), the column was eluted with PBS for 20 min at 0.25 ml min^{-1}. Subsequently, the composition was altered gradually from 100% PBS to the PBS–methanol mixture over 40 min. The flow rate during elution with PBS–methanol was 1 ml min^{-1}.

2.3. Sample preparation

Separate solutions of ESSE and of each thiol compound were prepared in PBS. The concentration of the ESSE solution was 4×10^{-4} M and the composition of each thiol solution was 2×10^{-4} M. Each thiol solution was mixed with ESSE in a ratio of 1:3 ensuring that the ESSE remained in excess. The mixtures were left for 20 min at ambient temperature to reach equilibrium. 75 μl of the solution was injected for each analysis.

2.4. Calibration

A 2×10^{-4} M glutathione solution was added to excess ESSE and after 20 min a 75 μl aliquot of the solution was injected into the column. This procedure was repeated 10 times on different days. The %R.S.D. for the 10 samples was calculated. Glutathione solutions at concentrations ranging from 1×10^{-6} M to 3×10^{-4} M were mixed with the stock solution of ESSE in such a

manner that the ESSE remained in excess. After equilibration 75 μl was removed for analysis from each mixture. The peak area of ES^- (λ_{max} 412 nm) and ESSG (λ_{max} 357 nm) were plotted against the corresponding glutathione concentration.

2.5. Reaction of ESSE with sodium hydroxide

A 2×10^{-4} M buffered solution of ESSE was mixed with 4 M sodium hydroxide in a 1:1 v/v mixture. The resulting solution had a pH of 14. After standing for 20 min and for 4 h respectively, aliquots of the solution were adjusted to a pH of 7.4 using orthophosphoric acid and the sample analysed using the HPLC method.

2.6. ^1H-NMR spectroscopy

The solution obtained by mixing 2 mg 0.5 ml^{-1} of Ellmans reagent with NaOD (pH 10) for 4 h was analysed by ^{1}H-NMR spectroscopy on a Bruker WM-250 using a standard ^{1}H-NMR menu (0–10 ppm) except that a 5 s relaxation delay was introduced to ensure that any distortion in the integrals was kept to a minimum.

3. Results and discussions

The reactions of Ellmans reagent and thiol compounds are well documented and depending on conditions a number of reactions are possible. Under the conditions used in this study the standard reactions shown below occur [17].

$$ESSE + RSH \rightleftarrows ESSR + ES^- + H^+ \quad (1)$$

$$RSSE + RSH \rightleftarrows RSSR + ES^- + H^+ \quad (2)$$

Provided ESSE was present in excess, only the reaction described by Eq. (1) occurs to any appreciable extent. This was the basis of the method of analysis proposed here and of many standard, biological and analytical procedures. The use of excess ESSE provided a method of pre-column derivatisation which allowed the separate identification of species in the chromatogram which were directly related to standard thiol estimations. Three separate signals were readily detected (Fig. 1). For example, on incubation of excess Ellmans reagent with glutathione (GSH), the Ellmans anion (ES^-) produced a peak with a retention time of 11 min and a maximum absorbance at 412 nm. The peak due to the mixed disulphide (ESSG) had a retention time of 36 min (Table 1) and a maximum absorbance at 357 nm and the Ellman's reagent peak had a retention time of 61 min and a maximum absorbance at 325 nm.

The peak due to ESSE was identified by passing Ellman's reagent down the column. The ES^- peak was identified by altering the quantity of thiol added and observing the change in the relative area of the ES^- and the ESSE peaks. The electronic spectrum of ES^- and ESSE have different absorption maxima (412 nm and 325 nm respectively). The mixed disulphides, however, have similar spectra. The peaks were identified by their spectra and the variable, thiol dependant position max.

This reaction scheme worked well with a number of different thiols and each component was well separated. The retention time of the mixed disulphide varied significantly from thiol to thiol (Table 1) but the absorption spectra of most of the mixed disulphides were very similar. One key advantage of the technique is its ability to distinguish between closely related compounds. For example, it is possible to distinguish between *N*-acetyl penicillamine and penicillamine and *N*-acetyl-cysteine and cysteine. Such discrimination could be of significant value in in vivo analysis. In the case of *N*-acetyl-cysteine and cysteine, *N*-acetyl-cysteine is cleaved by liver enzymes to provide cysteine to be transported into the cell where it can be used for glutathione synthesis [2]. The method proposed here can distinguish between cysteine, *N*-acetyl-cysteine and glutathione and has potential in studies of glutathione synthesis. Because the assay is based on the estimation of the mixed disulphides (ESSR) and excess ESSE is used, the assay is unaffected by aerobic oxidation during the pre-analysis period, which can be a problem when Ellmans anion (ES^-) is used.

To assess the use of the technique for quantitative analysis, a more detailed study was carried out for glutathione. A calibration graph is given in Fig. 2. The detection limit, based on $3 \times$ signal

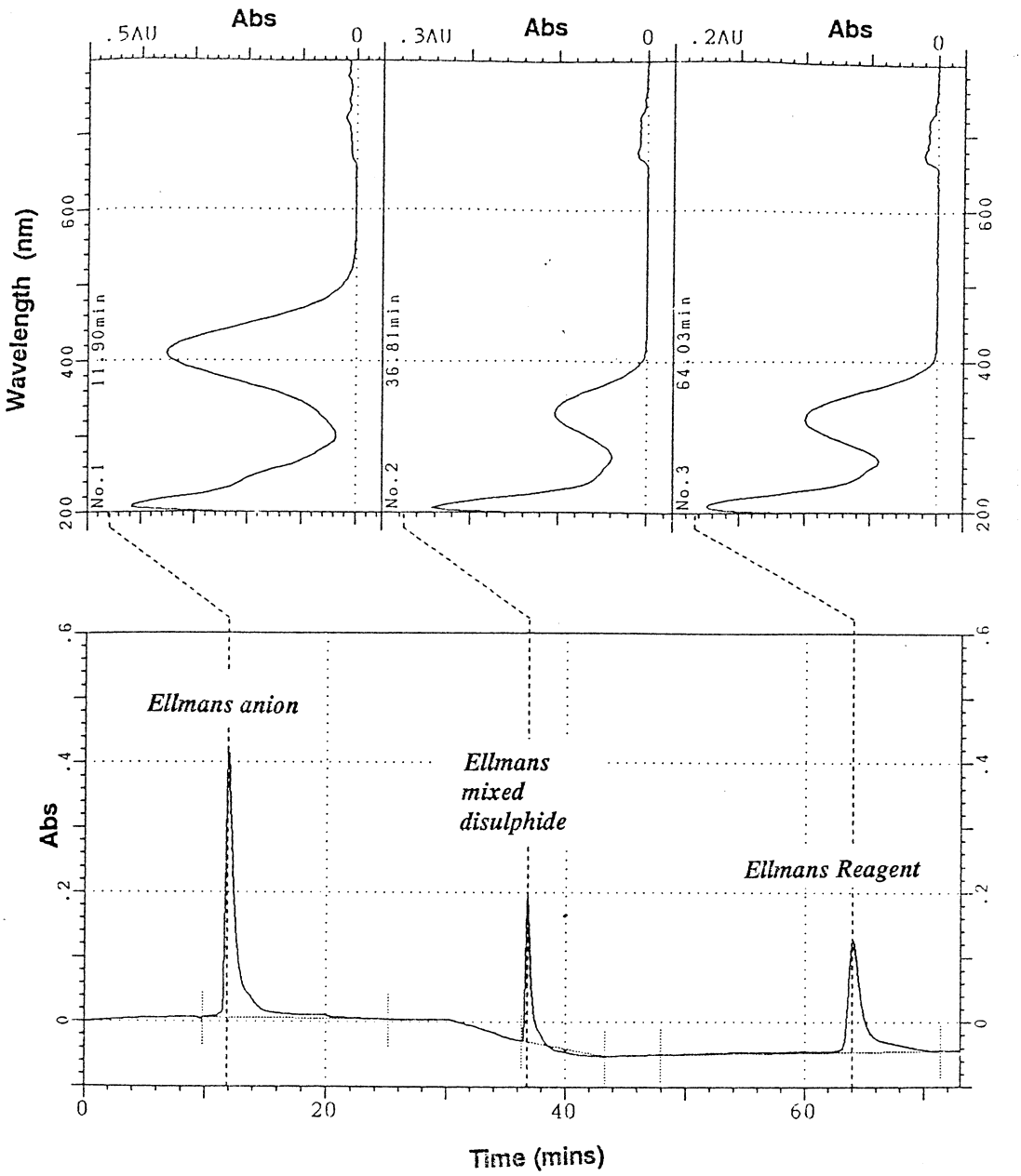

Fig. 1. Top: the 3-D chromatogram, wavelength (nm) vs. retention time (min) obtained following the derivatisation of glutathione by Ellmans reagent. Bottom: the corresponding spectrum index plots obtained from the derivatisation of glutathione.

to noise, was 1.0×10^{-5} M. In addition, ten different glutathione samples of the same concentration were estimated on different days to determine the %R.S.D. (1.3%). As the HPLC approach separates the mixed disulphide from the other components in the reaction mixture, it is possible to calculate the molar absorbtivity of ESSG ($\varepsilon_{ESSG} = 1450$ cm^2 mol^{-1} at 357 nm).

There have been reports that Ellman's Reagent can be oxidised under the conditions used in

Table 1
Retention times of the derivatised thiols (ESSR) detected at 357 nm

Compound	Retention time (min)
Thiomalic acid	21
Cysteine	30
Glutathione	36
N-acetyl-cysteine	41
D-Penicillamine	45
N-acetyl-penicillamine	56
Captopril	61

routine analysis [18]. Thus, the improper preparation of ESSE solutions can lead to incorrect quantitation. In the standard assay, the identification

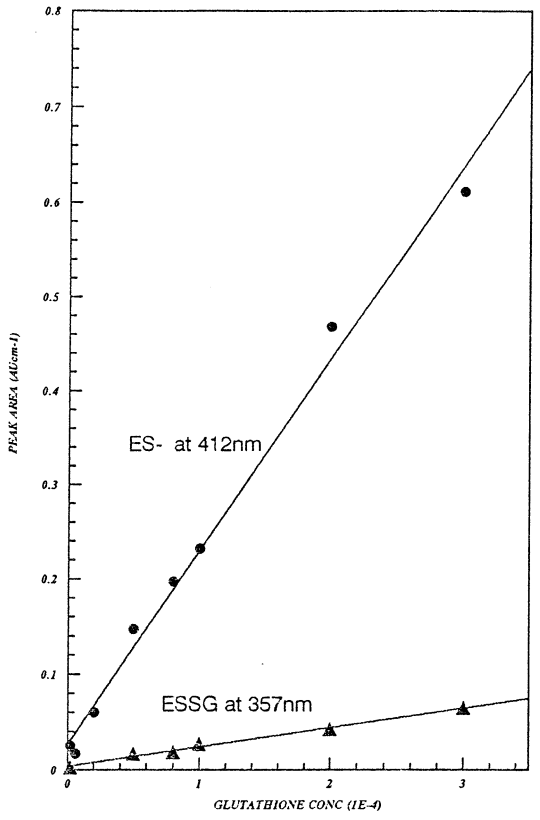

Fig. 2. The calibration graph for ESSG (at 357 nm) and ES^- (at 412 nm) derived from the reaction of excess Ellmans reagent with glutathione ($0–10^{-4}$ M). Each point is a duplicate injection. The equation of the line $(y) = 3.6928x + 0.3821$; correlation co-efficient $(R^2) = 0.9905$

of these impurities is very difficult. One method of inducing partial oxidation of Ellman's reagent is to incubate the reagent in alkaline conditions (Eqs. 3 and 4) [18,19].

$$2ESSE + 4NaOH \rightarrow 3ES^- + 4Na^+ + ESO_2^- + 2H_2O \quad (3)$$

$$ESSE + 2NaOH \rightarrow ES^- + 2Na^+ + ESO^- + H_2O \quad (4)$$

The question of whether the product of the reaction was ESO^- or ESO_2^- is more easily answered by NMR than by chromatography. ESSE was titrated with concentrated sodium hydroxide. At the end point equilibrium is reached at pH 10. The Ellman's anion and the oxidised product were clearly identified in the NMR spectrum (ES^-: doublet, δ7.75, 1H; doublet, δ7.3, 1H; singlet, δ7.2, 1H; ESO_2^-: doublet, δ8.15, 1H; doublet, δ7.7, 1H; singlet, δ7.6, 1H) and the ES^-: ESO_2^- ratio could be evaluated by simple integration of the corresponding resonances. The results showed that the anion was present as a 3:1 ratio of the oxidised product. This suggests that Eq. (3) is correct and the product is ESO_2^-.

The disproportion of ESSE in the presence of sodium hydroxide was not reversed by lowering pH, a step essential to protect the column before HPLC analysis with the proposed method. At a pH in excess of 8, an intense amber colour tends to be produced in the solutions used. This amber colour reduced to a yellow when the pH was reduced to pH 7 by the addition of orthophosphoric acid. In the chromatogram the appearance of a second peak with a retention time of approximately 14 min was found, which was assigned to ESO_2^- (Fig. 3). This assignment was confirmed by considering the time dependence of the reaction. In the above reaction, Ellman's anion should re-oxidise to form ESSE with time but the product ESO_2^- will not re-equilibrate. A sample was incubated for a period of 4 h rather than 20 min. The additional peak was clearly visible. Thus, one of the oxidation products of the unwanted side reactions known to occur with Ellman's reagent can be identified by this method of analysis. Despite careful preparation of the test solutions used in this study a peak at 14 min was

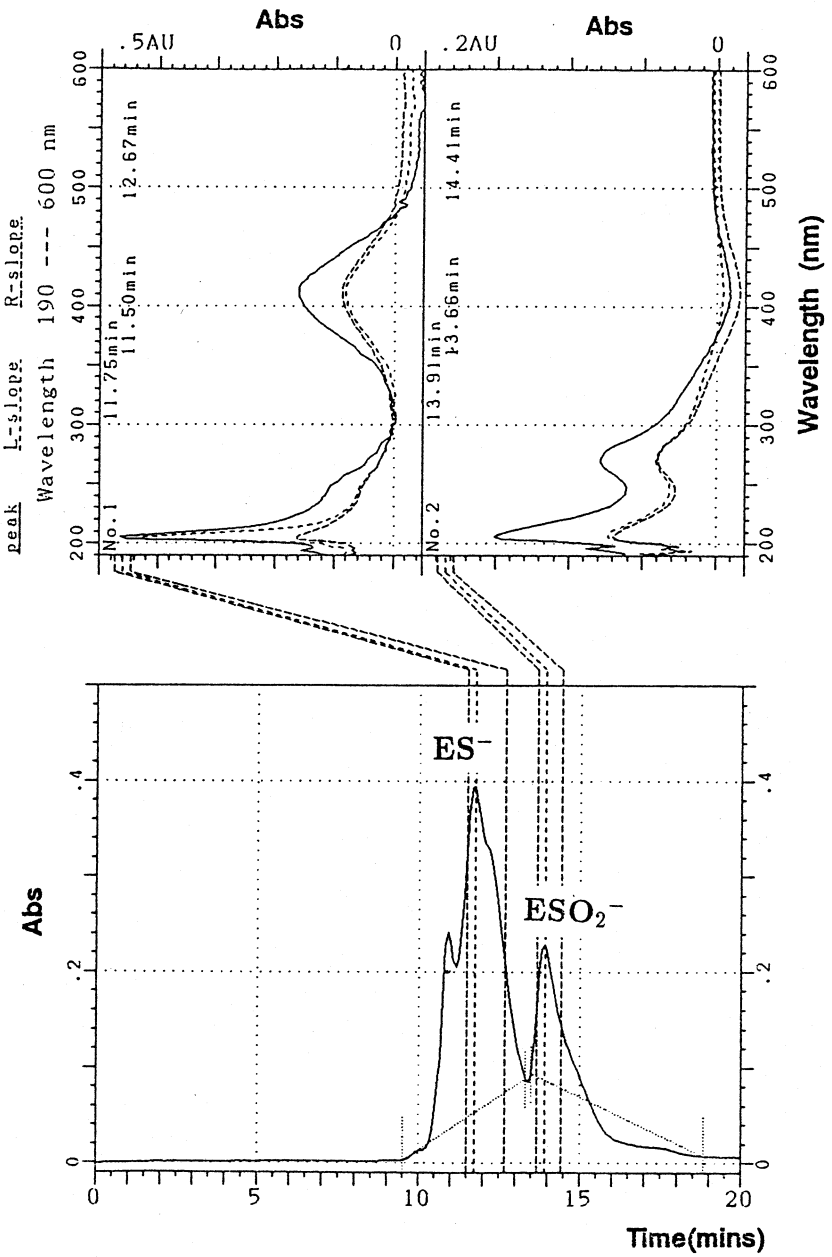

Fig. 3. The chromatogram and spectrum index plot obtained from the alkaline ESSE solution treated with orthophosphoric acid (pH 7.4)

observed on certain occasions. The experiments shown above indicate clearly that the reaction described in Eq. (3) was not reversible and that the oxidation product was retained. Such changes have been noted by a number of workers using Ellman's reagent [18,19]. However, of more importance in the use of ESSE in quantitative analysis is the fact that a consequence of the oxidation reaction three equivalents of ES^- are introduced into solution. Thus the overall affect is to create

an overestimation of the sulphydryl concentration (Eq. (3)).

The method developed above is valuable in providing a very great improvement in the security with which analysis using Ellman's reagent can be carried out. In addition it enables speciation in closely related thiol compounds and therefore can be used in analysis of metabolites in mixtures of closely related thiols. The method is quantitative and sensitive.

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