Determination of glutathione, cysteine and N-acetylcysteine in rabbit eye tissues using high-performance liquid chromatography and post-column derivatization with 5,5'-dithiobis(2-nitrobenzoic acid)

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Abstract

A high-performance liquid chromatographic method to determine glutathione, cysteine and N-acetylcysteine in rabbit retina, vitreous and lens has been developed. The thiols are separated using a 25×0.46-cm octadecylsilane column with 0.5 M phosphate buffer, pH 3, as mobile phase. The detection, at 412 nm, involves a post-column derivatization with 5,5,-dithiobis(2-nitrobenzoic acid) in presence of cationic micelles of hexadecyltrimethylammonium bromide that enhances the sensitivity. The detection limits are 0.21, 0.92 and 0.61 μmol/g wet sample for glutathione, cysteine and N-acetylcysteine, respectively.   © 1997 Elsevier Science B.V.

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1. Introduction

The tripeptide glutathione is the major intracellular non-protein-free thiol, which is found in a wide variety of biological tissues. Although it is present in several forms: reduced (GSH), oxidized (GSSG), and protein bound (PSSG), the GSH is by far the predominant form.

Among other important physiological functions, glutathione participates in the detoxification of drugs and the transport of amino acids by γ-glutamyl cycle, being a radioprotective agent and a free radical scavenger [1–3]. With respect to ocular tissues, it has been demonstrated that in lens the process of cataractogenesis in a number of experimental models corre-lates well with decreasing levels of GSH [4], and GSH also contributes to the maintenance of normal cornea hydration [5] and to the defense against oxidant damage of the retina [6]. Consequently the determination of GSH in ocular samples is almost routinely required.

The determination of aliphatic thiols is difficult due to the absence of a strong chromophore or fluorophore, so analytical methods involving a rapid conversion of the thiols to stable or measurable derivatives have been usually chosen. GSH can be perfectly determined by enzymatic assays [7–11], but high-performance liquid chromatography (HPLC) methods are often preferred because they allow the determination of other thiols in the same run. Reagents such as maleimides [12,13], phenyliso-thiocyanate [14], ethacrynic acid [15], o-phthalal-

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dehyde [16], 1,1'-[ethenylidenebis(sulfonyl)]bis-benzene [17], monobromo- [18–21] and monochlorobimane [22,23] have been applied to the precolumn derivatization in the HPLC analysis of GSH. Nevertheless, the post-column derivatization is frequently selected instead of pre-column derivatization because it has some advantages, such as taking place on-line with the HPLC system so the analytical method can be automated, and also it is not necessary to remove the excess of reagent. As a consequence of that, the total procedure can be shortened. In this way, a post-column procedure with on-line electrochemically generated bromine on a custom-made coulometric cell [24] has been proposed to evaluate GSH and GSSG in liver microsomes [25], and GSH, GSSG plus the protein-bound form (PSSG) in calf lenses [26].

The aim of this work was to examine the potential use of a well-known reagent: 5,5,-dithiobis(2-nitrobenzoic acid) (DTNB), for the post-column derivatization of GSH, cysteine and acetylcysteine using a conventional UV-visible detector. Although DTNB was used some years ago to determine thiols in general [27–31], it has not been used in GSH post-column derivatization yet. As cationic micelles of cetyltrimethylammonium have been recognized to improve the sensitivity in several determinations, involving sulfite and sulfide ions [32,33], in this work the influence of cationic micelles of hexadecyltrimethylammonium bromide (HTAB) in the post-column reaction has also been studied in an attempt to achieve lower detection limits.

Finally, the proposed method was applied to the analysis of rabbit eye tissue samples.

2. Experimental

2.1. Reagents

GSH, N-acetylcysteine, and cysteine standards were supplied by Sigma Aldrich Química (Madrid, Spain) and the standard solutions were prepared in 2 mM ethylenediaminetetraacetic acid (EDTA), pH 6. HTAB and CTBA were also purchased from Sigma, and their solutions were prepared in 0.5 M phosphate buffer. All other chemicals used, including eluent

solvents and 1,4-dithiothreitol (DTT) were analytical-reagent grade from Fluka (Buchs, Switzerland).

2.2. Apparatus and chromatographic conditions

Batch measurements were made on a Perkin-Elmer Lambda 3B UV–Vis spectrophotometer (Beaconsfield, UK) furnished with quartz cells of 1 cm pathlength.

The set-up used for continuous assays was composed of a Constametric III dual piston pump from Milton Roy (Riviera Beach, FL, USA) used to propel the chromogenic reagent (a solution containing 40 mg/l DTNB and 80 mg/l HTAB in 0.5 M phosphate buffer, pH 9), at a flow-rate of 0.25 ml/min. A Constametric 3500 dual piston pump from LDC Analytical (Riviera Beach, FL, USA) that was employed to propel the mobile phase (0.05 M phosphate buffer, pH 3) at a flow-rate of 1 ml/min. Samples were injected by means of a Rheodyne 7125 injector (Berkeley, CA, USA) with a fixed volume loop of 100 μl. The column used was of 25×0.46-cm I.D. packed with 5-μm particles of Spherisorb 5 ODS 2 from Phenomenex (Torrance, CA, USA). For detection, an SM4000 UV-Vis variable wavelength detector from Milton Roy set at 412 nm was used. Peak areas were measured using a JCL6000 Chromatography Data System from Jones Chromatography (Littlenton, CO, USA).

In order to develop the derivatization reaction, compounds eluted from the column were mixed with the reagent on a 7-m piece of PTFE tubing of 0.8 mm I.D.

For sample treatment, a 2.0 RS centrifuge from Omnifuge (Heraeus Sepatech, Germany) and an Ultrafree-MC 10.000 NMWL filter unit from Millipore Ibérica (Madrid, Spain) were used.

2.3. Animal study

The animals were maintained and used in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

A total of 20 New Zealand pigmented rabbits supplied by J. Córdoba Coop. (Valladolid, Spain) each weighing 1.5–2 kg were used. All operations were performed with the animal under general

anaesthesia by using a mix of intramuscular ketamine hydrochloride (35 mg kg^{-1} of body mass) and xylazine hydrochloride (3 mg kg^{-1} of body mass).

A group (G_1) of 10 rabbits was used as control group, and another group (G_2) of 10 rabbits was used to ascertain the influence of the use of N-acetylcysteine added to the irrigating solution in vitrectomy. Three hours after the vitrectomy the animals were killed by using intracardiac injection and samples of ocular tissues were taken.

2.4. Sample treatment

The different tissues (retina, vitreous and lens) were weighed (0.2 g of lens, 0.04 g of retina and 0.7 g of vitreous), homogenized and diluted in 2 ml of 2 mM EDTA, pH 6. Then, vitreous samples were sonicated for 10 s, and lens and retina for 30 s. Subsequently, 1.5 ml of the resultant mixtures were centrifuged at 2500 g and 4°C in eppendorf tubes, the supernatant was collected and frozen at −80°C until HPLC analysis. For the HPLC analysis, the samples were slowly unfrozen on ice, then passed through Ultrafree-MC 10.000 NMWL filter units for 45 min. The final solution was injected into the HPLC system. To determine GSSG, 0.2 ml of the samples were previously treated with 5 µl of 20 mM DTT solution.

3. Results and discussion

3.1. Batch study of the reaction

The product of the derivatization showed a high absorption, between 350 and 450 nm, with a maximum at 412 nm.

When the pH of the solution of DTNB was varied from pH 2 to 10, it was observed that the absorbance increased and that the maximum absorbance was obtained at pH near 8; so this value was selected.

Allowing GSH to react with different concentrations of DTNB in 0.5 M phosphate buffer, pH 8, it was observed that the absorbance increased gradually until it reached a constant level at DTNB concentrations above 12 mg l^{-1}. By assaying the other compounds we came to the conclusion that the best DTNB concentration to be used was 15 mg l^{-1}. This solution must be changed every 3 days.

The presence of HTAB in the reaction mixture increased the signal obtained for the three compounds. As can be seen in Fig. 1, when HTAB was used, the signal nearly doubled especially for GSH
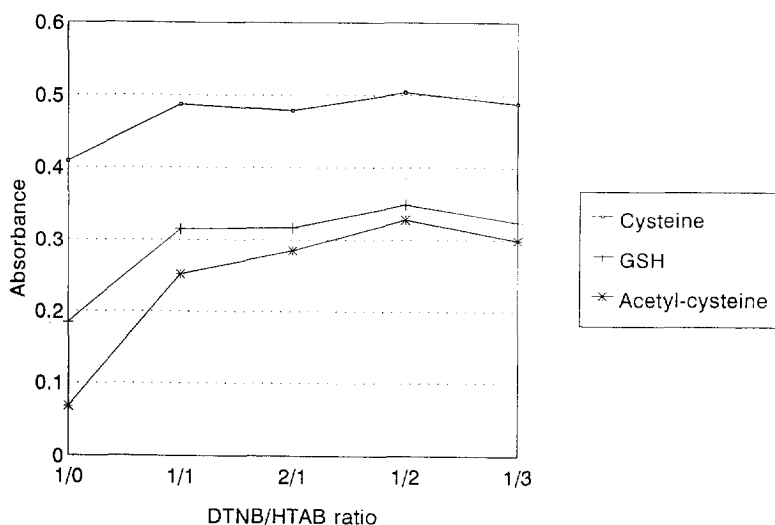

Fig. 1. Variation of the absorbance with the DTNB/HTAB ratio at a fixed DTNB concentration (15 mg l^{-1} in 0.5 M phosphate buffer, pH 8).
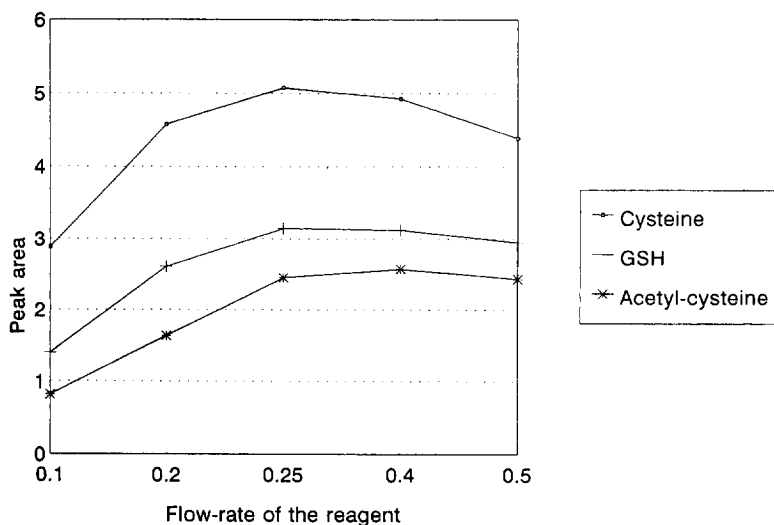
Fig. 2. Variation of the peak area with the flow-rate of the reagent.

and N-acetylcysteine. The maximum signal was obtained at a DTNB/HTAB ratio of 1:2.

3.2. Application of the reaction to post-column derivatization in HPLC

Once the optimal batch conditions for the reaction were established, they were applied to the post-column derivatization in order to evaluate GSH, cysteine and N-acetylcysteine by HPLC with detection at 412 nm.

The flow-rate of the mobile phase was fixed at 1 ml min^{-1} and, as can be seen in Fig. 2, the highest signals were obtained using a flow-rate of 0.25 ml min^{-1} for the reagent. The mixture was conveyed into a PTFE capillary of 7 m×0.8 mm I.D. These dimensions allowed a good mixture between reagent and eluate, providing a good baseline and also a small peak broadening.
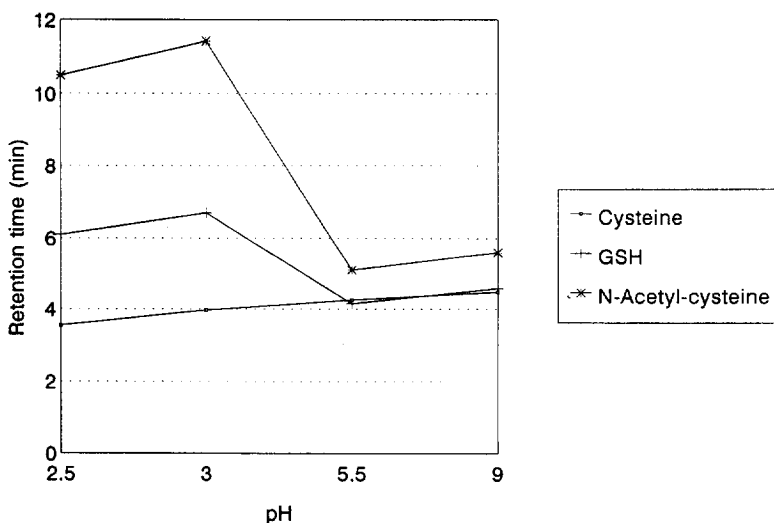

Fig. 3. Variation of the retention time with the pH of the mobile phase.

The mobile phase was 0.05 M phosphate buffer and, according to the results obtained when the pH of the mobile phase was varied (Fig. 3), the best separation was obtained at pH 3.

Due to the dilution resulting from joining the column eluate and the reagents, higher reagent concentration (40 mg l^{-1} DTNB and 80 mg l^{-1} HTAB) and higher pH (the reagents were dissolved in 0.5 M phosphate buffer, pH 9) were used.

The application of the proposed procedure to a model mixture (1.7 mg l^{-1} in each) provided good resolution (Fig. 4) and sensitive detection. The detection limits obtained according to the IUPAC were 19 µg l^{-1} GSH, 26 µg l^{-1} cysteine and 18 µg l^{-1} N-acetylcysteine, which allow the analysis of these compounds in samples at low concentration levels. The repeatability ($n=7$) and reproducibility ($n=7$ each day for 5 days) of the method were checked and the R.S.D. found ranged between 1.1 and 3.2% and between 2.1 and 5.6%, respectively.

3.3. Application to the analysis of eye tissues

Fig. 5 shows chromatograms of the three types of rabbit eye tissue: vitreous, retina and lens. As can be appreciated, there is no interference of the matrix and the compounds can be identified and quantified perfectly. When samples were treated with DTT, this compound eluted at 21 min, so the analysis time used was 30 min (Fig. 6).

Table 1 shows the results obtained (always on wet sample) in the analysis of single samples from the two groups. In Table 1 we can see the detection
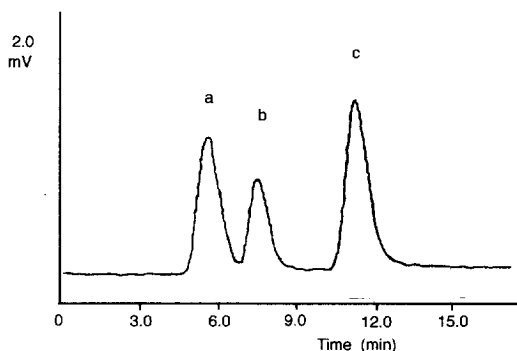

Fig. 4. Chromatogram of a standard mixture (1.7 mg l^{-1} in each): cysteine (a), GSH (b), N-acetylcysteine (c).
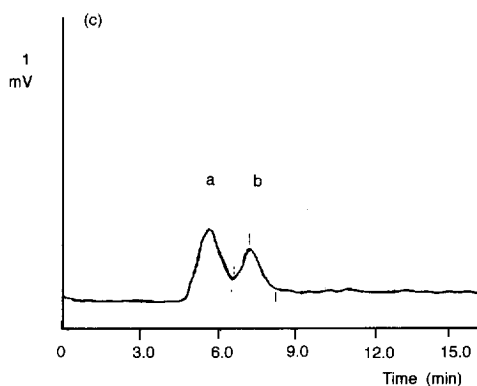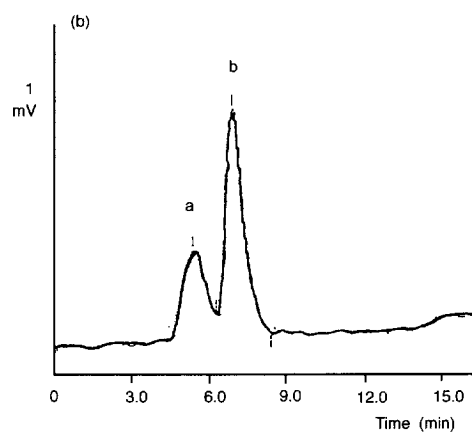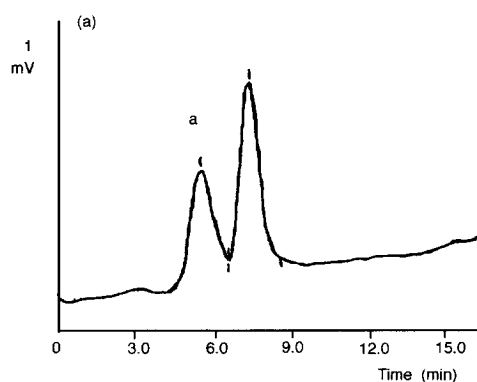
Fig. 5. Chromatograms of the different tissues: lens (a), retina (b) and vitreous (c). See Fig. 4 for peak identification.
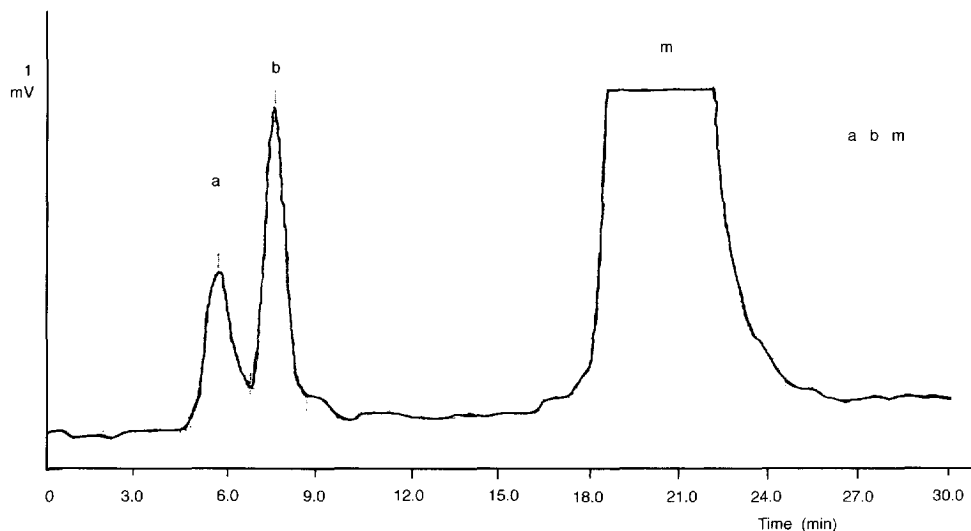
Fig. 6. Chromatogram of a retina sample treated with DTT (m). See Fig. 4 for peak identification.

limits, the extreme values of the range, the average and the relative standard deviation. It is important to note that in this case the R.S.D. values are very high in comparison with those ones obtained on model solutions, mainly when vitreous samples are analyzed. This may be related to the fact that the vitreous is considered as a depot, where some metabolic residues from the surrounding tissues, especially the retina, will be eliminated. So, residual quantities of N-acetylcysteine were found in all the vitreous samples from group G_2, whereas this compound was only detected in three retina samples and two lenses.

Due to the absence of data related to the vitreous and the great dispersion found in this work, we consider that the physicochemical characteristics of the vitreous might have a notable influence on the values obtained. This should be confirmed with more experimental and detailed studies on this kind of samples.

Table 1
Interval (μg/g wet sample), average and relative standard deviation obtained for the control group (G_1) and the group treated with N-acetylcysteine (G_2)

Compound	Retina		Vitreous		Lens	
	G_1	G_2	G_1	G_2	G_1	G_2
GSH						
Interval	6.58–13.97	0.86–14.79	0.46–3.06	1.01–7.30	6.29–10.78	8.44–12.10
Average	11.89	12.33	1.76	2.70	10.20	10.64
%R.S.D.	8.35	9.12	24.50	25.60	7.1	8.03
GSSG						
Interval	0.03–0.10	0.08–0.17	—[a]	0.09–0.18	0.11–1.03	0.20–1.73
Average	0.06	0.13	—	0.14	0.42	0.71
%R.S.D.	13.20	15.32	—	29.16	8.51	10.12
Cysteine						
Interval	—[a]	1.04–1.48	—[a]	1.14–3.26	0.94–1.60	1.94–3.35
Average	—	1.23	—	1.87	1.27	2.51
%R.S.D.	—	11.56	—	10.17	6.49	7.20

[a]Under the detection limit.

In group G_2, comprised of those rabbits whose vitrectomy was carried out using an irrigating solution with added N-acetylcysteine, it can be also observed that the values for GSH, GSSG and cysteine have increased.

4. Conclusions

The reaction between DTNB and thiols can be applied to the post-column derivatization of GSH, cysteine and N-acetylcysteine previously isolated by HPLC using a Spherisorb ODS 2 column.

The presence of cationic micelles of HTAB in the reaction mixture enhanced the sensitivity of the detection by nearly two-fold. The detection limits obtained with the post-column reaction ranged from 18 to 26 $\mu g\ 1^{-1}$, this allowed the analysis of the compounds at low concentration levels.

When the optimized HPLC post-column derivatization procedure was applied to analyze eye tissue samples, there was no interference of the matrix and the three compounds could be determined. These results make post-column derivatization with DTNB a very useful alternative to determine GSH, cysteine and N-acetylcysteine in eye tissues.

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