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## Note

# Method for determination of free and total glutathione and γ-glutamylcysteine concentrations in human leukocytes and plasma

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In animals, tissue concentrations of glutathione are normally millimolar, whereas the plasma level is in the micromolar range [1]. Similar results are found in humans, although the absolute levels for the compound may be lower [1–4]. This difference in concentration between plasma and the intracellular compartments is mainly due to the rapid extracellular clearance of glutathione [1], principally regulated by γ-glutamyl transpeptidase located on the external surface of the cell membrane [1].

Simple, sensitive and easily obtainable "tools" for studying glutathione metabolism in humans are critical aspects in experimental work on this biologically important and unique thiol-containing tripeptide [1, 2]. During fasting, decreased concentrations of glutathione are found in liver [5], intestine [5] and plasma [6] of rats, whereas the level in erythrocytes is essentially unaltered [5]. In humans, the level of leukocyte glutathione decreases during short-term starvation [4], and this cell type may thus be used as a sensitive indicator of glutathione and sulphur amino acid depletion [4].

This paper describes a thiol-specific clean-up procedure [7] and high-performance liquid chromatography (HPLC) with post-column *o*-phthaladehyde (OPA) derivatization that constitute a sensitive (pmol) and selective method. A quantitation of not only the leukocyte glutathione concentration [4], but also the γ-glutamylcyst(e)ine level, may thus be achieved. Since the sensitivity also permits the determination of the corresponding compounds in plasma, data on glutathione and γ-glutamyl-cysteine concentrations in plasma are also given. These "tools" should be useful in studies of glutathione metabolism.

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## EXPERIMENTAL

### *Apparatus*

The HPLC sysem consisted of a ConstaMetric III solvent-delivery pump (LDC, Riviera Beach, FL, U.S.A.), a Rheodyne Model 7120 (Berkeley, CA, U.S.A.) injection valve with a 100-$\mu$l loop, a 250×4.6 mm I.D. column (Supelco, Bellefonte, PA, U.S.A.) packed with 5-$\mu$m Spherisorb $C_{18}$ (Macherey-Nagel, Düren, F.R.G.), a 250×4.6 mm I.D. guard column packed with Polygosil 60 $C_{12}$ (particle size 25–40 $\mu$m), an Altex 100 A pump (Berkeley, CA, U.S.A.) with an internal pulse dampener for the post-column derivatization reagent, and a Fluoromonitor III (LDC) using an excitation wavelength of 350 nm and emission wavelength of 420 nm. A linear Model 255 potentiometer recorder was used to register the fluorescence.

### *Chemicals*

All chemicals used were analytical grade and all water was ultrapure water (Milli-Q-water system, Millipore, Bedford, MA, U.S.A.). Boric acid, methanol, phosphoric acid and sulphosalicylic acid were purchased from Merck (Darmstadt, F.R.G.). 2-Mercaptoethanol, mercaptoacetate (thioglycolic acid), dithiothreitol (DTT), OPA and glutathione (reduced form) were obtained from Sigma (St. Louis, MO, U.S.A.). 1-Heptansulphonate (sodium form) was from Fisons (Loughborough, U.K.). $\gamma$-Glutamylcysteine was prepared from bis-$\gamma$-L-glutamyl-L-cystine dihydrate (Vega Fox, Tucson, AZ, U.S.A.) after reduction with DTT. Standard solutions of glutathione and $\gamma$-glutamylcysteine were prepared in 0.05 *M* nitric acid containing 2 m*M* disodium EDTA. The ethyl acetate was water-saturated and prepared as previously described [8].

### *Sample preparation*

Four male and four female normal-weight healthy subjects aged 28–40 years participated in the study. Blood samples (heparinized) were taken at 09:00 a.m. after a 10-h fast, to avoid interference from the known circadian variation in free glutathione concentration [9]. Since no sex difference was observed, data from both sexes were pooled.

Leukocyte (10 ml of whole blood) and plasma (2 ml) samples for free and total glutathione and $\gamma$-glutamylcysteine determinations were prepared essentially as previously described for glutathione [4], except that cell lysis was performed prior to incubation with DTT for total glutathione and $\gamma$-glutamylcysteine analyses. The leukocyte suspension was then allowed to stand for 30 min before the sulphosalicylic acid was added [4]. After an initial centrifugation at 2500 *g* for 2 min to remove blood cells [4], plasma was treated with DTT (final concentration 2 m*M*) for 30 min. Sulphosalicylic acid (final concentration 0.12 *M*) was then added. Leukocyte and plasma samples were then allowed to stand for 25 min at 4°C. After centrifugation, all samples were analysed immediately. If stored for more than a week (−70°C), a steady decline in $\gamma$-glutamylcysteine concentration was found. For glutathione the disappearance was not as pronounced, but a decrease to 70% of original level was observed after five months. All solutions

used also contained disodium EDTA (final concentration 1 m*M*) and serine borate (final concentration 20 m*M*).

*Analytical procedure*

The selectivity of the method [10] was based on the use of an organomercurial adsorbent (*p*-acetoxy-mercurianiline-Sepharose 4-B, PAMAS) with a high affinity (7 μmol/ml) for thiol compounds [7]. Since DTT also shows affinity for the PAMAS column, and was present in high concentrations in all the total glutathione samples, it had to be eliminated. This was achieved by ethyl acetate extraction (3×3 volumes) prior to the PAMAS step [11]. To ensure complete removal of all ethyl acetate, these samples were kept at 40°C with a continuous flow of nitrogen for 20 min. Sulphosalicylic acid did not cause deterioration of the PAMAS column. Finally, it was essential for a good recovery of both glutathione (99–102%) and γ-glutamylcysteine (94–97%) that the sample was kept at a pH below 2.0 [12], since rapid loss of both compounds was otherwise seen.

The leukocyte and plasma samples were applied on a 0.1-ml PAMAS column, pre-washed with 2.0 ml of 50 m*M* nitric acid. After the sample had been applied the column was washed with 1.0 ml of 50 m*M* nitric acid. The compounds were then eluted with 0.50 ml of 25 m*M* mercaptoacetic acid in 50 m*M* nitric acid containing 1 m*M* disodium EDTA. The mobile phase was prepared as follows: to a solution of 1.0 l of 5 m*M* sodium heptanesulphonate, concentrated phosphoric acid was added to adjust the pH to 1.8. This solution was filtered through a 0.45-μm filter and deaerated, and methanol was added to a concentration of 11% (v/v). The effluent rate was 1.2 ml/min. The separation column had to be washed with methanol–water (40:60, v/v) after each day's use to prevent rapid deterioration and extend the column lifetime to more than two months, during which 300 samples could be analysed without loss of efficiency. The OPA reagent was prepared according to Friedman et al. [13], with minor modifications [14]. The flow-rate was kept at 1.0 ml/min.

Owing to the thiol content of glutathione and γ-glutamylcysteine, both compounds spontaneously react with OPA [15, 16]. The presence of mercaptoacetate serves a dual purpose, as a reducing agent [17] and, in synergy with mercaptoethanol, as a thiol activator to improve the sensitivity and rate of the OPA reaction. Sample determinations referring to free glutathione or γ-glutamylcysteine refer only to the free reduced form of the compound, whereas total concentrations refer to the reduced free and oxidized (symmetric or mixed disulphide) forms, plus any protein-bound forms of the compounds. A limitation of the present method is that cysteine does not react optimally with OPA [18] and thus cannot be measured. The free oxidized (disulphide) form of GSH (GSSG) was not quantified, but could be after a simple modification of the procedure. Since GSSG does not bind to PAMAS, the PAMAS washings of a free GSH sample contained this compound. When this fraction was reduced with DTT, as in the procedure for total GSH determination, and reset on a PAMAS column, GSSG could be measured.

## RESULTS AND DISCUSSION

Preliminary experiments using the original clean-up procedure for glutathione [4] showed a poor recovery. The result may partly have been due to the use of hydrochloric acid in the AG-50 step [4], since chloride ions presumably complexed with the mercury ions of the PAMAS adsorbent. Substitution of nitric acid abolished this effect. However, the recovery for glutathione over the AG-50 column was only 60–70%. Increasing the concentration of the nitric acid solution to 4.0 *M* did not improve the recovery. An alternative method of eliminating DTT using ethyl acetate extraction was then tried and found to be satisfactory. A slight reduction of sample volume was observed, but this was not considered critical because standards and samples were treated in parallel and the whole sample volume was applied on the PAMAS column. Interestingly, sulphosalicylic acid of the present concentration was not found to affect the PAMAS adsorbent and could be ignored. In the final procedure the original AG-50 step [4] was excluded, and samples for free glutathione determination were applied directly on the PAMAS column, whereas samples for total glutathione determination were first extracted with ethyl acetate.

The addition of EDTA to all solutions (1 m*M* final concentration) was essential to prevent oxidation of the compounds [1]. The low pH of the mobile phase was critical to achieve complete protonation of all carboxyl groups of the glutathione molecule and thereby an acceptable separation of the two compounds (Fig. 1). The $pK_a$ value of the most acidic group is ca. 2.2, but we chose to set the pH of the mobile phase to 1.8 since a rapid deterioration of the chromatographic column was seen below. The column material used (Spherisorb) was found more resistant than Nucleosil (Macherey-Nagel) in this respect.

Acceptable recoveries were found for both compounds (five samples): $98 \pm 2$ and $96 \pm 2\%$ (mean $\pm$ S.D.) for reduced and total glutathione, respectively. For $\gamma$-glutamylcysteine, the corresponding values were $96 \pm 2$ and $92 \pm 3\%$, respectively. Inter-assay (five samples) variations of 6.4 and 10.1% for glutathione and $\gamma$-glutamylcysteine, respectively, were calculated. A linear standard plot was obtained between 20 pmol and 2 nmol for GSH. The detection limit (signal-to-noise ratio 3) was 4.5 pmol, calculated as previously described [14]. Normal fasting leukocyte and plasma concentrations of glutathione and $\gamma$-glutamylcysteine are given in Table 1. Unexpectedly, the total and free glutathione values for leukocytes and plasma were only ca. 75 and 85% of those previously published [4], using the Tietze technique [19]. The percentage of free glutathione (97%) in leukocytes using the present technique, however, was of the same order as for most other tissues [1], rather than the corresponding value (85%) in our previous report [4]. In plasma, in accordance with a previous report [20], ca. 75% was in the free reduced form, expressing the more "oxidative milieu" of plasma [1]. Interfering compounds were not observed in the chromatogram, reflecting the specificity of the clean-up procedure. A potential compound must contain both a free thiol and an amino group. The two most likely compounds that normally occur in measureable amounts are cysteine and cysteinylglycine. Both cys-

TABLE I

NORMAL FASTING LEUKOCYTE AND PLASMA FREE REDUCED AND TOTAL GLUTATHIONE AND γ-GLUTAMYLCYST(E)INE CONCENTRATIONS

Values are mean ± S.D.; $n = 8$.

| Compound | Leukocyte (pmol per $10^6$ cells) | Plasma ($\mu M$) |
|---|---|---|
| Glutathione (reduced) | 1670 ± 138 | 4.98 ± 0.65 |
| Glutathione (total) | 1730 ± 145 | 6.65 ± 0.51 |
| γ-Glutamylcysteine (reduced) | 3.3 ± 0.6 | 1.7 ± 0.2 |
| γ-Glutamylcysteine (total) | 4.6 ± 0.5 | 3.1 ± 0.3 |

teine [18] and cysteinylglycine (unpublished data), however, react poorly with OPA.

Information on tissue or plasma levels of γ-glutamylcysteine is sparse [1, 3], owing to the generally low concentration of the compound. It is interesting to note that the levels of glutathione and γ-glutamylcysteine that we found in leukocytes and plasma are of the same order as those previously published for erythrocytes and plasma [3]. These findings confirm observations made in vitro, and in animals, of a rapid clearance of plasma glutathione [1], whereas the leukocyte

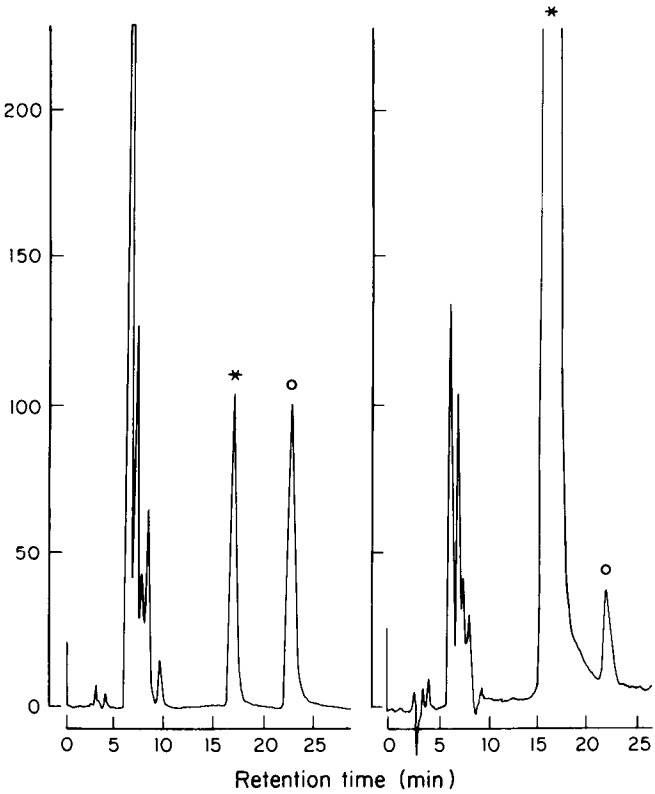


Fig. 1. Standards (200 pmol, left-hand side) and unspiked leukocyte (diluted 1:1 with mobile phase) sample for free reduced glutathione (*) and γ-glutamylcysteine (○). Fluorimeter sensitivity set at $K = 5$. All other experimental details are given in Experimental.

level is much higher, 4–6 m*M* (calculated on a mean corpuscular volume of 200 fl) and of a similar order to other tissues with high glutathione levels, e.g.liver and kidney [1]. γ-Glutamylcysteine, on the other hand, does not seem to accumulate intracellularly to the same degree (ca. 5–10 μ*M*). Our observations on γ-glutamylcysteine support earlier data of a low intracellular concentration and "tight" regulation of the compound [1], principally evoked through glutathione synthetase, γ-glutamylcyclotransferase and negative inhibition of its synthesis regulated by the intracellular glutathione level [1]. Extracellular γ-glutamylcysts(e)ine has recently been found to be efficiently transported into the cell [1], thereby generating an alternative pathway for glutathione synthesis. This pathway may be a route for cells with limited γ-glutamylcysteine synthetase activity to synthesize glutathione [1]. It is interesting to note that cells fed γ-glutamylcysteine may generate above normal glutathione levels [1]. The importance of the latter pathway in leukocyte glutathione metabolism has not yet been investigated, but might be of interest when there is a therapeutic aim to reduce intracellular levels of glutathione by inhibition of γ-glutamylcysteine synthetase [1].

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REFERENCES

1 A. Meister and M.E. Anderson, Ann. Rev. Biochem., 52 (1983) 711.
2 A. Larsson, S. Orrenius, A. Holmgren and B. Mannervik (Editors), Function of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects, Raven Press, New York, 1983.
3 L. Hagenfelt, A. Arvidsson and A. Larsson, Clin. Chim. Acta, 85 (1978) 167.
4 J. Mårtensson, Metabolism, 35 (1986) 118.
5 E.S. Cho, N. Sahyoun and L.D. Stegink, J. Nutr., 111 (1981) 914.
6 M.E. Anderson and A. Meister, J. Biol. Chem., 255 (1980) 9530.
7 U.Hannestad and B. Sörbo, Clin. Chim. Acta, 95 (1979) 189.
8 J. Mårtensson, Anal. Biochem., 154 (1986) 43.
9 J.T. Issacs and F. Binkley, Biochim. Biophys. Acta, 498 (1977) 29.
10 L.C. Mokrasch and E.J. Teschke, Anal. Biochem., 140 (1984) 506.
11 J. Butler, S.P. Spielberg and J.D. Schulman, Anal. Biochem., 75 (1976) 674.
12 D. Perret and S.R. Rudge, J. Pharm. Biomed. Anal., 3 (1983) 3.
13 Z. Friedman, H.W. Smith and W.S. Hancock, J. Chromatogr., 182 (1980) 414.
14 E. Andersson, E. Hånkansson, J. Larsson and J. Mårtensson, J. Chromatogr., 414 (1987) 174.
15 V.H. Cohn and J. Lyle, Anal. Biochem., 14 (1966) 434.
16 P.G. Richman and A. Meister, J. Biol. Chem., 250 (1975) 1422.
17 R.A. Houghten and C.H. Li, Methods Enzymol., 91 (1983) 554.
18 M. Roth, Anal. Chem., 43 (1971) 880.
19 F. Tietze, Anal. Biochem., 27 (1969) 502.
20 O.W. Griffith, Anal. Biochem., 106 (1980) 207.