Journal of Chromatography, 382 (1986) 253—257
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3249

Note

Sensitive measurement of glutathione using isocratic high-performance liquid chromatography with fluorescence detection

N.K. BURTON and G.W. AHERNE*

Division of Clinical Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH (U.K.)

(First received March 14th, 1986; revised manuscript received May 5th, 1986)

Glutathione (GSH) is the major non-protein sulphydryl in cells. It performs many major roles in cellular metabolism including detoxification, biosynthesis and transport. Large amounts of GSH are found in the liver, the major drug metabolising organ, and GSH has been found to affect the efficacy or toxicity of many drugs [1].

Many methods have been used to quantify GSH although not all of them have proved satisfactory. The original methods were total non-protein thiol assays which were highly non-specific [2]. More specific assays were developed by the use of enzymatic methods. These included the use of glyoxylase [3] and glutathione reductase [4] which are stoichiometric reactions. Sensitivity was enhanced by the use of recycling assays, e.g. utilising the oxidation of NADPH by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) which is catalysed by GSH and GSH reductase [5, 6].

Recently, high-performance liquid chromatography (HPLC) techniques have been developed which allow rapid, sensitive and selective measurement of GSH. These include systems utilising electrochemical [7], colorimetric [8] and fluorescence [9] detection. Biological thiols can be derivatised prior to HPLC with monobromobimane (mBBr) to form fluorescent adducts [10, 11]. Monobromobimane derivatisation was used by Minchinton [12] using an HPLC method for GSH. Disadvantages of this HPLC method for GSH are the long sample turnover time (> 20 min) and the need to use a gradient system. A fast and sensitive method using monobromobimane derivatisation with an isocratic HPLC separation has therefore been developed.

0378-4347/86/$03.50

EXPERIMENTAL

Materials

GSH, oxidised glutathione (GSSG), DL-buthionine-(*S*,*R*)-sulphoximine (BSO), N-acetyl cysteine (NAC), cysteine (Cys) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma (Poole, U.K.). Metaphosphoric acid (MPA), ammonium hydrogen carbonate, ethylenediaminetetraacetic acid (EDTA), boric acid, potassium chloride, sodium hydroxide and ammonium dihydrogen orthophosphate were laboratory grade purchased from BDH (Eastleigh, U.K.). Hydrochloric acid and glacial acetic acid were analytical grade, methanol was HPLC grade and all were obtained from May and Baker (Dagenham, U.K.). Tetrabutylammonium hydrogen sulphate was purchased from Aldrich (Gillingham, U.K.) and mBBr was obtained from C.P. Labs. (Bishops Stortford, U.K.). All water used was double glass distilled.

Stock mBBR solutions at a concentration of 250 μg/ml in acetonitrile were stored at −20°C.

Method

The dilution buffer for standards and samples consisted of two volumes of 10 m*M* ammonium hydrogen carbonate, 1 m*M* EDTA pH 8.0 and one volume of 15% MPA having a final pH of 1.8. The borate buffer was prepared from 0.2 *M* potassium chloride and 0.2 *M* boric acid with the pH being adjusted to 10.5 using 0.2 *M* sodium hydroxide.

For the measurement of GSH in plasma, blood was collected into cooled (4°C) EDTA blood tubes and separated as soon as possible after withdrawal. Plasma (200 μl) was added to 100 μl of 15% MPA, vortex-mixed and centrifuged for 2 min at 9950 *g* in an Eppendorf 5412 bench centrifuge (Anderman, East Molesley, U.K.). The supernatant was decanted and stored at −20°C until assayed.

Tissues were freeze-clamped immediately on withdrawal from the animal and the tissue was stored under liquid nitrogen until needed. Tissue (1 g) was homogenized with 3 ml of 15% MPA in a PTFE glass homogeniser driven by an electric motor (approx. 3500 rpm) until the tissue was disrupted and the homogenate was centrifuged at 9950 *g* for 2 min. The supernatant was decanted and stored at −70°C until analysed. The supernatant was diluted with dilution buffer to obtain a concentration within the range of the standard curve before assaying.

The plasma supernatant or diluted tissue supernatant (20 μl) was added to 180 μl of 0.2 *M* borate buffer pH 10.5 and 10 μl of 250 μg/ml mBBr were added. The mixture was incubated for 30 min at room temperature in the dark and 10 μl glacial acetic acid were added to stop the reaction. The samples were then stored on ice in the dark until 50 μl were injected onto the column. The samples at this stage are stable, on ice, for approximately 24 h but can be stored for three days in the dark at −20°C.

The chromatographic procedure was performed using a Kipp Analytica 9208 pump (MSE Scientific Instruments, Crawley, U.K.) connected to a Waters 420 AC fluorescence detector (Waters Assoc., Harrow, U.K.) equipped with a 395-nm band-pass excitation filter and a 455-nm emission filter. Separation was

achieved using a 150 × 5 mm, 3-μm Hypersil-ODS column (HETP, Macclesfield, U.K.). The mobile phase consisted of methanol—50 m*M* ammonium dihydrogen orthophosphate (30:70) containing 10 m*M* tetrabutylammonium hydrogen sulphate and adjusted to pH 5.9 with solid ammonium hydrogen carbonate. The flow-rate was 1 ml/min with a back-pressure of 10 MPa. GSH standards were made up in the buffer which was used to dilute the tissue homogenate and the standard curve was obtained by plotting GSH—mBBr adduct peak height against GSH concentration.

Liver GSH levels were measured in Schneider random bred mice (30—35 *g*) injected with one intraperitoneal injection of 0.5 ml of either saline (0.9%), BSO (2 mmol/kg in 0.9% saline) or NAC (2 mmol/kg in 0.9% saline).

Treatment was carried out between 09:00 and 09:30 h. The animals were sacrificed 4 h after administration and the liver, kidneys, heart and lungs were immediately freeze-clamped and stored under liquid nitrogen until needed. Homogenates (25%) were prepared and stored as described above.

RESULTS AND DISCUSSION

Typical chromatograms illustrating the separation of GSH standards and tissue extracts are shown in Fig. 1. The retention time of GSH is 6.3 min and it is well separated from other peaks. Sample turnover is about 11 min.

The peak was confirmed to be GSH by incubating red blood cells for 1 h with excess CDNB before extraction. This treatment caused the peak to completely disappear (Fig. 2). CDNB reacts specifically with GSH in cells to form a conjugate via glutathione S-transferases.

Over the range of the standard curve (50—250 pmol added to column) the response of the detector was linear (equation of line $y = 0.566x - 3.88$, correlation coefficient = 0.9997) with coefficients of variation given in Table I. Outside this range the response is non-linear. There were no significant differ-

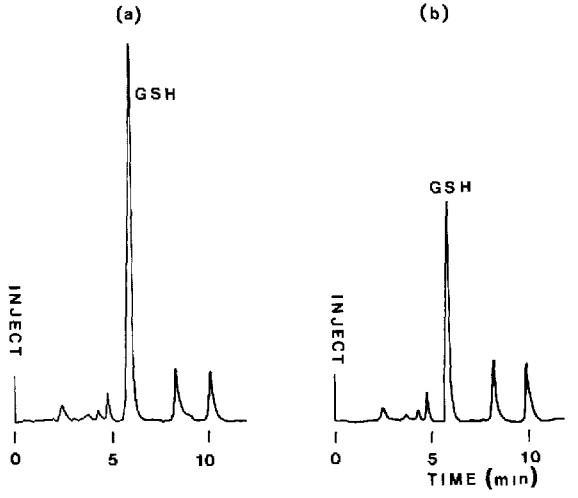

Fig. 1. Typical chromatograms of (a) 200 pmol GSH added to column and (b) liver homogenate (1:100 dilution). Both chromatograms at detector sensitivity of ×16.

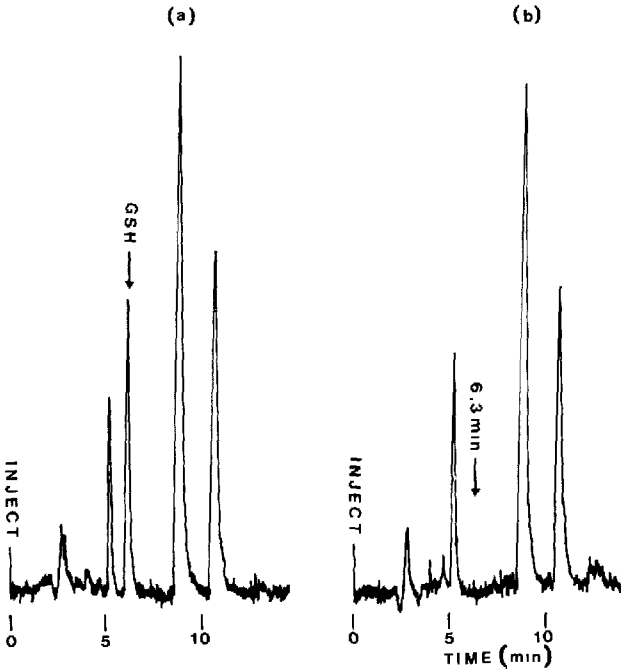

Fig. 2. Chromatograms of (a) whole blood extract (1:100 dilution) and (b) extract of whole blood after treatment with CDNB (1:10 dilution). Both chromatograms at detector sensitivity of ×128.

TABLE I

MEAN OF SIX STANDARD CURVES SHOWING STANDARDS DEVIATIONS (S.D.) AND COEFFICIENTS OF VARIATION (C.V.)

Amount of GSH added to column (pmol)	Mean peak height (mm)	S.D.	C.V. (%)
250	137.2	5.1	3.7
200	109.6	5.5	5.0
150	79.9	3.2	4.1
100	51 0	2.5	4.9
50	26.5	2.1	8.0

ences between the standard curve fitted to the quadratic or to the linear function within the above range.

By preparing the standards in an acidic medium (pH 1.8) oxidation is minimised. Comparison of standards prepared in acidic and neutral solution showed that 40.9 ± 3.8% (n=4) was lost after 30—60 min at room temperature in a pH 7.0 buffer. Standards prepared in acidic buffer showed no loss even after storing for 24 h at 4°C and after three weeks the loss was 13%. Samples prepared and stored in acidic conditions, i.e. homogenisation with 15% MPA, showed a loss of 25.02 ± 4.36% (n=4) after one month at —20°C. We suggest, therefore, that samples should be measured within two weeks of homogenisation unless stored at temperatures below —70°C.

The intra-assay variation for samples was 3.4% (n=5). There was no interference from either Cys or GSSG.

The effect of BSO, a γ-glutamyl cysteine synthetase inhibitor, and NAC, a GSH precursor, on mouse tissue GSH levels was measured using the described assay. There was no significant difference in the liver, heart or lung levels of GSH 4 h after treatment with BSO or NAC as compared with saline-treated animals. Kidney GSH levels were significantly lower ($P < 0.001$) 4 h after administration of BSO. Eighteen hours after treatment with BSO, GSH levels in kidney, heart and lung were significantly lower than saline-treated controls (Table II).

TABLE II

TISSUE GSH LEVELS 4 OR 18 h AFTER ADMINISTRATION OF SALINE, BSO AND NAC

	GSH concentration (mean ± S.D., n = 5) (μmol/g of tissue)			
	Liver	Kidney	Heart	Lung
Saline	6.74 ± 0.54	4.41 ± 0.34	0.92 ± 0.29	1.99 ± 0.18
BSO (4 h)	5.14 ± 0.88	0.70 ± 0.18 (P<0.001)*	0.72 ± 0.20	1.71 ± 0.28
BSO (18 h)	6.58 ± 1.69	2.48 ± 0.66 (P<0.001)*	0.48 ± 0.06 (P<0.02)*	1.29 ± 0.24 (P<0.005)*
NAC (4 h)	7.70 ± 1.17	4.35 ± 1.62	1.15 ± 0.32	2.32 ± 0.37

*Significant differences in GSH tissue levels from saline-treated animals.

The method described gives adequate sensitivity for the measurement of plasma GSH concentrations. Plasma GSH concentrations were measured in five normal human volunteers following an overnight fast and 1 h after a meal. Mean values of 3.0 ± 0.35 nmol/ml and 2.48 ± 0.30 nmol/ml ($P < 0.025$) were obtained, and agree with other published values [13].

In view of the recent upsurge of interest in the metabolic roles of GSH a sensitive, specific and rapid method for its determination in biological samples is required. The method described in this paper incorporates these features with equipment which is simple and inexpensive with no requirement for gradient programmers or sophisticated detectors. In addition, with slight modifications of the method, the possibility exists for the simultaneous measurement of other biological thiols, e.g. γ-glutamyl cysteine and cysteine.

REFERENCES

1 B.A. Arrick and C.F. Nathan, Cancer Res., 44 (1984) 4224.
2 V.H. Cohn and J. Lyle, Anal. Biochem., 14 (1966) 434.
3 G.E. Woodward, J. Biol. Chem., 109 (1935) 1.
4 E. Bernt and H.U. Bergermeyer, Meth. Enzym. Anal., 4 (1974) 1643.
5 F. Tieze, Anal. Biochem., 27 (1969) 502.
6 J.E. Brehe and H.B. Burch, Anal. Biochem., 74 (1976) 189.
7 E.G. Demaster, F.N. Shirota, B. Redfern, D.J.W. Goon and H.T. Nagasawa, J. Chromatogr., 308 (1984) 83.
8 A.J. Baars and D.D. Breimer, Pharm. Weekbl. (Sci.), 5 (1983) 145.
9 R.C. Fahey, G.L. Newton, R. Dorian and E.M. Kosower, Anal. Biochem., 111 (1981) 357.
10 G.L. Newton, R. Dorian and R.C. Fahey, Anal. Biochem., 114 (1981) 383.
11 N.K. Burton, G.W. Aherne and V. Marks, J. Chromatogr., 309 (1984) 409.
12 A.I. Minchinton, Int. J. Rad. Oncol. Biol. Phys., 10 (1984) 1503.
13 E. Beutler and T. Gelbert, J. Lab. Clin. Med., 105 (1985) 581.