Journal of Chromatography, 194 (1980) 424–428
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,774

Note

Estimation of glutathione in rat liver by reversed-phase high-performance liquid chromatography: separation from cysteine and γ-glutamylcysteine

J. REEVE and J. KUHLENKAMP
Gastroenterology Section, Medical and Research Services, Wadsworth Veterans Administration Medical Center and The University of California, Los Angeles, CA (U.S.A.)
and
N. KAPLOWITZ*
691/111C, Wadsworth VA Hospital, Los Angeles, CA 90073 (U.S.A.)
(First received December 28th, 1979; revised manuscript received February 22nd, 1980)

Endogenous thiol peptides such as cysteine, γ-glutamylcysteine and glutathione are generally determined non-specifically in protein-free acid extracts by their reaction as a group with the Ellman reagent (5,5′-dithiobis(2-nitrobenzoic acid)) to form a mixed disulfide liberating a product measured at 412 nM[1]. Methods with greater specificity for glutathione involve enzymatic reactions (such as glutathione reductase)[2,3] or fluorometric adducts with *o*-phthalaldehyde[4]. However, all methods have the disadvantage of not quantitatively separating the three endogenous thiols in a direct fashion. We have modified the reaction of these sulfhydryl compounds with Ellman reagent to a high-performance liquid chromatographic (HPLC) method which quantitatively separates derivatives of the three compounds. The thiols are first reduced, then reacted with Ellman's reagent to form mixed disulfides which are separated and quantitated by peak height in HPLC. This method should be readily adaptible to the determination of specific radioactive and glutathione turnover studies and the enzymatic assays for the enzymes of glutathione synthesis.

METHODS

High-performance liquid chromatography

An Altex Scientific (Berkeley, CA, U.S.A.) Model 312MP liquid chromatograph equipped with a 210 sample injector and an Altex Hitachi 155-40 variable-wavelength detector was employed. All separations were performed on a LiChrosorb RP-18 column (10 μm, 250 × 4.6 mm) also from Altex Scientific. 20-μl samples were injected.

All substances were eluted with ammonium formate (0.023 M, pH 5)–methanol (90:10), at a constant flow-rate of 2 ml/min. The ammonium formate was filtered through a 0.5-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and degassed for 10 min under vacuum. Methanol was used directly. All runs were performed at room temperature.

0021-9673/80/0000–0000/$02.25 © 1980 Elsevier Scientific Publishing Company

REAGENTS AND CHEMICALS

Ammonium hydroxide and formic acid were from Mallinckrodt (St. Louis, MO, U.S.A.); HPLC-grade water was from Baker (Phillipsburg, NJ, U.S.A.) and glass-distilled methanol was from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Cysteine, reduced glutathione (GSH), and oxidized glutathione (GSSG), coenzyme A, dithioerythritol (DTE), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH and glutathione reductase were obtained from Sigma (St. Louis, MO, U.S.A.). γ-Glutamylcysteine was prepared from GSSG[5]. All reagents and thiol compounds gave single peaks in HPLC. The concentration of thiol compound standards was confirmed with a routine spectrophotometric method[1].

Tissue preparation

20% homogenates of rat liver were prepared in 0.01 *M* sodium phosphate–0.25 *M* sucrose pH 7.4 buffer. To 4 ml of the homogenate, 4 ml 8% sulfosalicylic acid (SSA) was added, giving a 10% homogenate in 4% SSA. Extract was centrifuged at 2500 *g* for 15 min and 0.051 ml of 10 *N* NaOH was added to 2.0 ml of the supernatant giving a pH of approximately 8.0. DTE (0.05 ml of a 200 m*M* solution) was added to the neutralized extract and incubated at room temperature for 30 min. DTNB (0.2 ml of 200 m*M* solution) was then added, yielding a final 8.75% homogenate; 20 μl of the mixture was used for HPLC. Thiol mixed disulfides with Ellman's reagent were monitored in the effluent at 280 nm.

Preparation of standards

To 2-ml aliquots of standards of cysteine, γ-glutamylcysteine, and glutathione in 0.1 *M* sodium phosphate pH 8.0, 0.05 ml DTE and 0.2 ml DTNB was added as above and 20 μl of the resultant solution used for HPLC.

Recovery experiments

The recovery of glutathione was determined by adding slightly greater than physiological concentrations to liver extracts and then determining by HPLC the change of glutathione in the liver extract. All recovery experiments were with liver extracts from four different rats. Glutathione was determined also by the method of Cohn and Lyle[4] and GSSG by oxidation of NADPH in the presence of glutathione[6].

RESULTS

A representative chromatogram demonstrating the separation of cysteine, γ-glutamylcysteine and glutathione is shown in Fig. 1. The retention times were 3.5, 4.8 and 6.6 min respectively, and were highly reproducible. GSSG (unreduced) and the coenzyme A product appeared in the solvent front. Standard curves for peak height (A_{280}) *versus* concentration of the three compounds is shown in Fig. 2. The lines were determined by least squares regression. A typical liver homogenate is shown in Fig. 3. Note that the sample was injected as a final 8.75% homogenate. Studies of the reproducibility of a single sample and variation of multiple analyses of different single samples demonstrate a high degree of reproducibility (Table I). Total glutathione (8.2 μmole/g) concentrations are in close agreement with determinations by

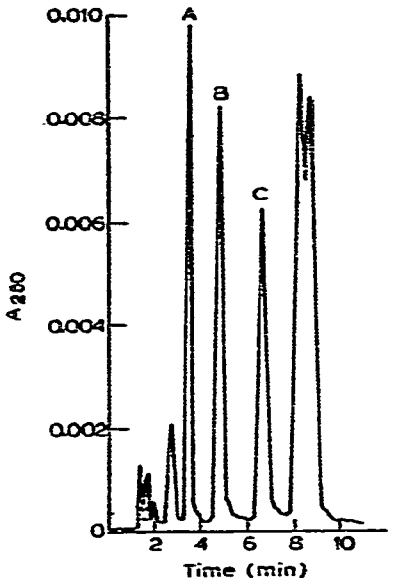

Fig. 1. Chromatogram of a mixture of thiol standards. Ordinate represents A_{280} and abscissa retention time. The concentrations of the respective standards were: cysteine (A, 0.1 m*M*), γ-glutamylcysteine (B, 0.075 m*M*) and glutathione (C, 0.075 m*M*).

an independent method using Ellman's reagent and NADPH glutathione reductase for total glutathione (8.1 μmole/g). Recovery of γ-glutamylcysteine (1 μmole/g) and glutathione (3 μmole/g) added in the physiologic concentration range to liver samples was 97 and 98% respectively. We are not aware of published γ-glutamylcysteine concentrations in liver and, therefore, cannot be certain of the homogeneity of the substance in that peak. The cysteine peak in liver sample is suspected of being

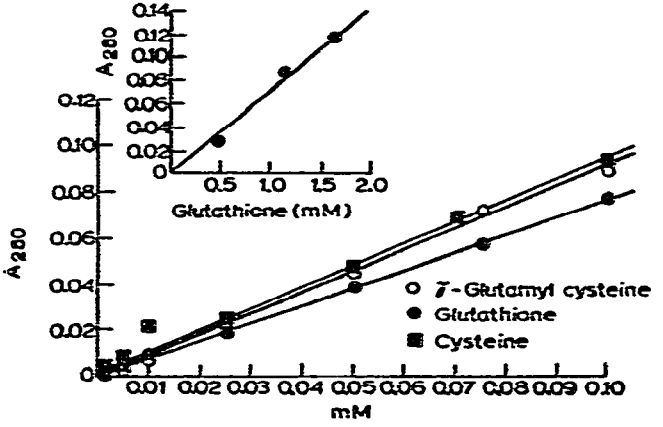

Fig. 2. Standard curves for A_{280} peak heights *versus* concentration of cysteine, γ-glutamylcysteine and glutathione. Insert represents peak height *versus* glutathione concentration in the physiologic range for liver (8.75% homogenate). The thiol concentrations on the abscissa were determined directly by a previously reported method and represent concentrations in starting material.

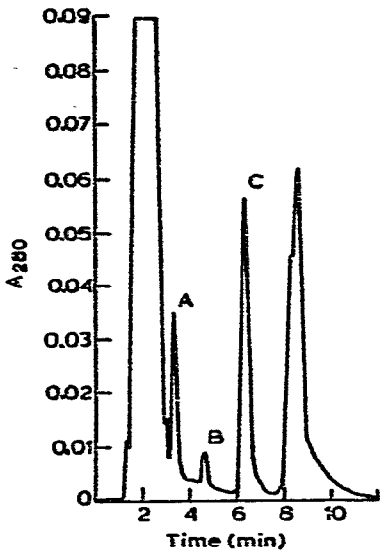

Fig. 3. Representative chromatogram of endogenous thiol peptides in 8.75% liver homogenate.

heterogenous since the values are an order of magnitude greater than published. Therefore, we did not pursue recovery experiments. Furthermore the cysteine peak was often obliterated by the solvent front in biologic samples. The coefficient of variation of hepatic glutathione determinations (four determinations) was 1.2% whereas it was 9.3% for hepatic γ-glutamylcysteine.

TABLE I

DETERMINATION OF TOTAL γ-GLUTAMYLCYSTEINE AND GLUTATHIONE IN RAT LIVER

Rat No.	A_{280}*	*Total GSH*** *concentration by HPLC (μmoles/g)*	*Total GSH**** *concentration (μmoles/g)*
1	0.073	8.6	8.8
2	0.070	8.3	8.6
3	0.063	7.6	7.3
4	0.069	8.1	7.8
Means ± S.D.	0.069 ± 0.004	8.2 ± 0.4	8.1 ± 0.7
		*Total γ-glutamylcysteine** concentration (μmoles/g)*	
1	0.011	0.86	
2	0.010	0.79	
3	0.008	0.61	
4	0.007	0.53	
Means ± S.D.	0.009 ± 0.002	0.70 ± 0.15	

* Absorbances with 8.75% homogenate injected into HPLC.
** These concentrations are corrected for dilution of liver samples.
*** Total GSH = reduced GSH + 2× GSSG (see Methods)

DISCUSSION

Our data demonstrate that cysteine, γ-glutamylcysteine and glutathione mixed disulfides with Ellman's reagent can be separated and quantitated by reversed-phase HPLC. The determinations are highly reproducible and the recovery of standards added to liver homogenates is excellent. We are aware of two other reports of HPLC methods for determining glutathione; both involve ion-exchange with electrochemical detection, one with a mercury-detector electrode[7] and the other with a graphite paste detector[8]. With the latter method glutathione in liver was one half of the literature value. No attempt was made to reduce oxidized glutathione or account for γ-glutamylcysteine.

Glutathione is synthesized in sequence from constituent amino acids by two enzymes: γ-glutamylcysteine synthetase (rate-limiting) and glutathione synthetase[9]. This method of separation of the precursor, cysteine, the intermediate, γ-glutamylcysteine, and the product, glutathione should offer a very useful approach to the determination of enzymatic activities for glutathione synthesis *in vitro*. Moreover, another very important application of this method currently in progress in our laboratory is glutathione turnover studies which involve determination of radioactive precursor incorporation into intermediates and products and degradation of glutathione *in vivo*. Specific radioactivity and pool size of glutathione precursors can be determined from HPLC with liquid scintillation spectrometry of collected effluents fractions.

ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Grant GM/CA-24987 and VA Medical Research funds. We wish to thank Ms. Anita Boesman for her expert secretarial support.

REFERENCES

1 N. Kaplowitz, *J. Pharmacol. Exp. Ther.*, 200 (1977) 479.
2 F. Teitze, *Anal. Biochem.*, 27 (1969) 502.
3 C. Crowley, B. Gillham and M. D. Thorn, *Biochem. Med.*, 13 (1975) 287.
4 V. H. Cohn and J. Lyle, *Anal. Biochem.*, 14 (1966) 434.
5 D. Strumeyer and K. Bloch, *Biochem. Prep.*, 9 (1962) 52.
6 N. Kaplowitz, J. Kuhlenkamp, L. Goldstein and J. Reeve, *J. Pharmacol. Exp. Ther.*, 212 (1980) 240.
7 D. L. Rabenstein and R. Saetre, *Anal. Chem.*, 49 (1977) 1036.
8 I. Mefford and R. N. Adams, *Life Sci.*, 23 (1978) 1167.
9 P. G. Richman and A. Meister, *J. Biol. Chem.*, 250 (1975) 1422.