Journal of Chromatography, 424 (1988) 141–146
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3951

Note

Measurement of plasma thiols after derivatization with monobromobimane

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(First received April 2nd, 1987; revised manuscript September 4th, 1987)

Thiols such as cysteine (Cys), glutathione (GSH) and homocysteine (Hcy) play a critical role in many biochemical pathways, but the difficulty of accurately measuring these compounds has hampered investigation of the role that they play in human disease. Measurement of thiols is difficult because they are highly reactive and easily oxidized during sample preparation. A number of assays are available for measurement of plasma thiols that are based either on the rate of an enzymatic reaction or the UV absorbance of a derivatized product. However, simultaneous measurement of Cys, GSH and Hcy in plasma has not been achieved using these techniques. This goal has been approached using electrochemical detection following chromatographic separation of the thiols [1–4]; however, selectivity and stability, reproducibility of the electrode are sometimes a problem [2,3].

The ability of monobromobimane (MBB) to react with thiol groups has been known for some time [5]. MBB is of particular interest because the bimane derivatives are fluorescent, permitting greater sensitivity of detection than assays based on UV absorption. Recently Fahey and co-workers [6–8] demonstrated that this reagent could be reacted with thiols and used for their quantitation. Derivatization is rapid and simple, and separation and quantitation can be done by high-performance liquid chromatography (HPLC). We have applied this assay to the measurement of Cys, GSH and Hcy in human plasma, defining coefficients of variation, recoveries and stability of these thiols in plasma, and identifying a good internal standard.

0378-4347/88/$03.50

EXPERIMENTAL

Chemicals

MBB [3,7-dimethyl-4-bromomethyl-6-methyl-1,5-diazabicyclo-(3.3.0)-octa-3,6-diene-2,8-dione] was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). Cys, GSH, Hcy and *d*-penicillamine were obtained from Sigma (St. Louis, MO, U.S.,A.). HPLC-grade methanol and sodium hydroxide were purchased from Fisher Scientific (Pittsburg, PA, U.S.A.) and acetic acid from Mallinckrodt (St. Louis, MO, U.S.A.). A stock solution of 10 m*M* MBB was made up in undiluted acetonitrile and stored in an amber bottle in the dark at −20°C. The stock solutions of Cys, GSH and Hcy were made up at 1 m*M* in outdated bloodbank plasma ultrafiltrate and stored at −20°C. A stock solution of *d*-penicillamine was made up at 10 m*M* in distilled water and stored at −4°C.

Samples

Blood was collected in tubes containing EDTA as an anticoagulant, and immediately centrifuged at 1000 *g* at 4°C for 10 min to remove cells and platelets. Plasma (1 volume) was then mixed with 0.4 volume of 10 m*M* MBB stock solution, 0.01 volume of 1 *M* acetic acid and 2.5 volumes of acetonitrile. In experiments comparing recovery, 2.5 volumes of 5% perchloric acid, 4% trichloroacetic acid or 6% sulfosalicylic acid was substituted for the acetonitrile. The mixture was then immediately centrifuged at 1000 *g* at 4°C for 10 min. The supernatant was filtered through a 5-μm membrane filter contained in a hypodermic needle (Monoject No. 305, Monoject, St. Louis, MO, U.S.A.). Aliquots (100 μl) were then analysed by HPLC. Outdated bloodbank plasma, deproteinized by filtration through an Amicon CF 25 filter (Amicon, Danvers, MA, U.S.A.), was used as a vehicle for reference compounds.

Instrumentation

The HPLC analyses were carried out with a Waters instrument (Waters, Milford, MA, U.S.A.) consisting of a U6K injector coupled to an M45 and a 6000A pump and a Model 660 gradient generator (used for washing the column between injections). The detector was a Spectroflow Model 980 programmable fluorescence detector (Kratos Analytical, Ramsey, NJ, U.S.A.) operating at an excitation wavelength of 365 nm with a 418-nm emission filter. A Model 440 integrator/plotter was used for integration of peak area. Solvent A consisted of 10% methanol (v/v) and 0.25% glacial acetic acid (v/v) in distilled water. Solvent B was 90% methanol (v/v) and 0.25% glacial acetic acid (v/v). The pH of both solvents was adjusted to 3.9 with sodium hydroxide. The μBondapak C_{18} radial compression reversed-phase column (Waters) was eluted isocratically with solvent A–solvent B (85:15) at a flow-rate of 1.5 ml/min. After each injection, the column was washed for 10 min with undiluted methanol at a flow-rate of 4 ml/min to remove late-eluting MBB hydrolysis products.

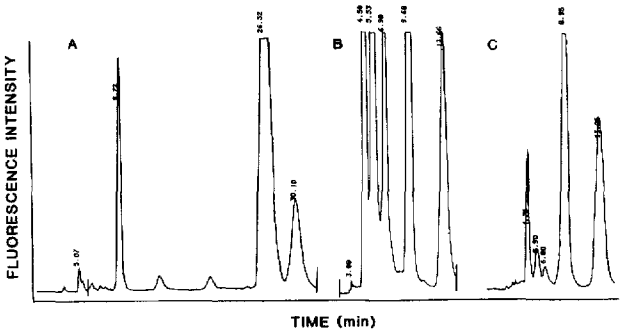

Fig. 1. (A) Chromatogam of MBB added to outdated bloodbank plasma ultrafiltrate; peaks at 9.72, 26.52 and 30.10 min are putative hydrolysis products. (B) Chromatogram of the same sample as on the left, with the addition of Cys, GSH and Hcy at 10 μM each plus 1 mM *d*-penicillamine; the peak at 4.50 min is Cys, 5.53 is GSH, 6.90 is Hcy, 9.68 is a hydrolysis product and 13.66 is *d*-penicillamine. (C) Chromatogram of a fresh plasma sample from a cancer patient; the peak at 4.75 min is Cys, 5.90 is GSH, 6.80 is Hcy, 8.95 is a hydrolysis product and 13.05 is *d*-penicillamine.

RESULTS

Chromatographic analysis of plasma ultrafiltrate prepared from outdated bloodbank plasma from which all Cys, GSH and Hcy had been removed by autoxidation demonstrated peaks eluting at a mean ($\pm$S.D.) of 9.65 ± 0.06, 26.50 ± 0.08 and 30.05 ± 0.08 min which increased slowly with time after addition of MBB (Fig. 1, left). These were thought to be hydrolysis products since the same peaks appear after the addition of MBB to water. When Cys, GSH and Hcy were added to the same plasma ultrafiltrates to a final concentration of 50 μM, and the internal standard *d*-penicillamine was added to a concentration of 1 mM, reasonably good separations of the thiol and putative hydrolysis products were achieved by isocratic elution (Fig. 1, right). The mean ($\pm$S.D.) retention times for a series of ten injections were 4.60 ± 0.15 min for Cys, 5.50 ± 0.10 min for GSH, 6.80 ± 0.10 min for Hcy, and 13.60 ± 0.06 min for *d*-penicillamine.

The detector response was linear over the concentration range between 10 and 500 nM for all three thiols when the detector was set at 0.005 a.u.f.s. The limit of detection was 10 nM for all three thiols in deproteinized plasma. An injection of 100 μl of deproteinized plasma containing 10 nM Hcy produced a peak height of 10 mm at 0.005 a.u.f.s. The coefficient of variation over the range 50–500 nM averaged 7.6% for Cys, 9.0% for GSH and 5.7% for Hcy.

The rate of reaction of MBB with plasma thiols appeared to be rapid, having reached completion by the end of the 10-min centrifugation at 4°C. During the derivatization reaction with MBB the pH of the plasma sample was found to be 7.4. The bimane derivatives of the three thiols were stable over a period of at least 2.5 h when the samples were kept at 4°C in the dark. The stock solutions of Cys, GSH, and Hcy stored at −20°C in air demonstrated no detectable loss in concentration over a fourteen-day period, facilitating their use for measurement of thiol concentrations in clinical samples.

The recovery of Cys, GSH and Hcy from plasma was investigated by comparing

TABLE I

COMPARISON OF THE RECOVERIES OF Cys, GSH, AND Hcy OBTAINED WITH DIFFERENT METHODS OF PLASMA DEPROTEINIZATION AS A FUNCTION OF THE RECOVERY OBTAINED WITH ACETONITRILE

Each result is the mean (±S.D.) of three experiments.

Method	Recovery relative to acetonitrile (%)			
	Cys	GSH	Hcy	*d*-Penicillamine
Sulfosalicylic acid	14±4	11 ±3	37±5	33±7
Perchloric acid	1±1	0.3±0.1	3±1	3±2
Trichloroacetic acid	1±1	0.3±0.1	5±3	22±21

the concentration measured for 100 μM standards made up in aged whole blood-bank plasma to standards made up in ultrafiltrates prepared from the same plasma. Samples were processed by adding acetonitrile to both the plasma and ultrafiltrate immediately after adding the thiols to a final concentration of 100 μM, and all the sample manipulations were carried out at 0°C. The recovery (mean ± S.D.) was 79±5.1% for Cys, 79±5.8% for GSH and 76±6.2% for Hcy (n=3). Dithiothreitol, 2-mercaptoethanol and ethanethiol proved to be unsuitable as internal standards either because of long retention times, broad peaks, or instability of the bimane derivative. On the other hand, *d*-penicillamine yielded a well defined peak with a mean (±S.D.) retention time of 13.60±0.76 min, the *d*-penicillamine–MBB derivative was stable for at least 120 min. Its recovery from freshly drawn whole plasma was 71±3.2% (mean ± S.D.). Loss of *d*-penicillamine due to oxidation was in the same range as for the other thiols studied (vide infra).

Table I shows a comparison of the recovery obtained using several different methods of deproteinizing plasma relative to the recovery obtained with acetonitrile-induced protein precipitation. These experiments were done using each of the thiols and the internal standard at a concentration of 100 μM. It is apparent that the use of acetonitrile produced the best recovery for the thiols of interest as well as for the internal standard.

Thiols are easily oxidized when they are exposed to the air. We investigated the stability of Cys, GSH and Hcy in aged bloodbank plasma in contact with air at 37°C using thiol standards at a final concentration of 100 μM and using acetonitrile for deproteinization. The half-lives were determined from measurements made at 15-min intervals for 1 h. In various samples the half-life ranged from 90 to 136 min for Cys, 17 to 60 min for GSH, 78 to 140 min for Hcy and 64 to 97 min for *d*-penicillamine.

Using *d*-penicillamine as an internal standard, the concentration of Cys, GSH and Hcy was measured in the plasma of ten patients with advanced (stage III) ovarian cancer. Blood samples were gently drawn into ice-cold tubes so as to minimize the risk of hemolysis; they were centrifuged immediately at 4°C to remove cells and platelets, and then processed on ice for derivatization. The mean (±S.D.) plasma concentrations were 2.7±1.8, 1.0±0.8 and 1.8±1.2 μM for Cys, GSH and Hcy, respectively.

DISCUSSION

There are two main problems in measuring the plasma concentration of Cys, GSH and Hcy. The first is that there is rapid loss of thiol compounds during sample preparation. This is probably due to the oxidative formation of both free and protein-bound disulfides [9,10]. The second is that the concentration of thiols in human plasma is very low, and most previously available assays do not have sufficient sensitivity to reliably quantify these compounds. The assay described in this report approaches the first problem by using an agent that reacts rapidly with thiols forming relatively stable derivatives. It deals with the second problem by using an agent that produces fluorescent derivatives which are detectable at much lower concentrations than derivatives that depend on ultraviolet absorption for their detection.

The major advantages of the MBB assay are, first, that it is capable of quantifying Cys, GSH and Hcy simultaneously in a single analysis. Secondly, it uses standard HPLC equipment readily available in most pharmacology laboratories. Thirdly, it permits immediate derivatization of the thiols so that it minimizes artifact formation due to oxidation during sample preparation. Fourth, it is specific for thiols and sufficiently sensitive. It can detect Cys, GSH and Hcy even at concentrations as low as 10 n*M*. Thus thiols in human plasma can be quantified. Fifth, the variance of the assay, both within and between samples and days, is small. Finally, the assay avoids the use of ultrafiltration, or precipiation with perchloric, trichloroacetic and sulfosalicylic acids. During assay development we found that significant amounts of all three thiols were lost if the samples were deproteinated by filtration through ultrafiltration membranes (Amicon CF-25 cones or Centrifree filters), or if they were deproteinated by precipitation with acid. The low recovery following deproteination with acid may be due to the fact that thiols react more slowly with MBB at low pH.

The HPLC analysis of the MBB–thiol derivatives was complicated by the appearance of several late-eluting peaks when MBB was added to aqueous solutions. While there was good resolution of these putative hydrolysis products from the Cys, GSH and Hcy peaks, it required substantial washing between injections to be sure that all of these compounds had eluted from the column.

Several investigators have noted that GSH is rapidly lost from biologic fluids including plasma [9–11]. The mechanism by which this occurs is not fully understood, but clearly involves oxidation of GSH to form both GSH disulfide and mixed disulfides. In rat plasma this process is mediated, at least in part, by a nondializable factor [9]. Previous studies have shown that loss of GSH can be slowed by acidification, but not by chelation with EDTA or EGTA [10]. Our results using aged plasma demonstrate loss of more than 50% of GSH in 1 h, and a somewhat slower loss of Cys, Hcy and *d*-penicillamine. The implication of these results for the clinical use of this or any assay for plasma thiols is that samples should be derivatized as quickly as possible. An additional concern is the fact the erythrocytes contain GSH at concentrations three orders of magnitude higher than the concentrations found in plasma. Thus hemolysis must be scrupulously avoided in order to obtain accurate measurements of plasma GSH.

The ideal internal standard for the measurement of plasma thiols would have the same recovery and stability as the plasma thiols of interest. The recovery of *d*-penicillamine was very close to that of Cys, GSH and Hcy, and the stability of *d*-penicillamine in plasma was in the same range as that of Cys and Hcy, although somewhat greater than that of GSH.

The values that we obtained for Cys, GSH and Hcy in ten ovarian cancer patients were somewhat lower than values reported for these three thiols by other investigators [12–19], probably reflecting the ability of this assay to measure only plasma thiols and not the disulfides resulting from their oxidation. It should now be possible to define the changes in these three plasma thiols associated with good health and various disease states.

ACKNOWLEDGEMENTS

We would like to thank Dr. Paul Andrews and Michael Murphy for their advice. We would also like to thank Steve Cleary and the nursing staff of the UCSD Medical Center for their assistance in obtaining plasma samples. This work was supported by Grants T3209290 and CA 23100 from the National Institutes of Health. This work was conducted in part by the Clayton Foundation for Research, California Division. Dr. Howell is a Clayton Foundation investigator.

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