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DETERMINATION OF PENICILLAMINE, PENICILLAMINE DISULFIDE AND PENICILLAMINE–GLUTATHIONE MIXED DISULFIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Methodology is described for the simultaneous determination of D-penicillamine, penicillamine disulfide and the penicillamine–glutathione mixed disulfide, as well as glutathione and glutathione disulfide, in human plasma, erythrocytes and urine. The various thiols and disulfides are separated by reversed-phase ion-pairing liquid chromatography with detection by an electrochemical detector with dual gold/mercury amalgam electrodes in series. The thiols are detected at the downstream electrode; the disulfides are reduced at the upstream electrode and then detected as the thiols at the downstream electrode. Detection limits (at a signal-to-noise ratio of 2.0) are in the picomole range for 20 μl of injected solution for all compounds except penicillamine disulfide, which has a detection limit of 600 pmol in 20 μl. A convenient method is described for preparation of the penicillamine–glutathione mixed disulfide by thiol/disulfide exchange with standardization of the solution by ^{1}H NMR spectroscopy.

INTRODUCTION

D-Penicillamine (D-PSH; β,β-dimethylcysteine) is used as a medicinal agent for the treatment of Wilson's disease [1], cystinuria [2], lead poisoning [3–5] and rheumatoid arthritis [6–9]. Recently, it also has been reported that D-PSH selectively inhibits the replication of human immunodeficiency virus, the acquired immune deficiency syndrome (AIDS) infective agent [10–12].

Although D-PSH has been used as a medicinal agent since 1954, it is only recently that it can be reliably assayed in biological fluids [13,14]. The devel-

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opment of a high-performance liquid chromatography (HPLC) method using electrochemical detection (ED) for the determination of D-PSH [14] and subsequent modifications of the method [15–17] have made possible studies of D-PSH pharmacokinetics [17–19]. However, the metabolism of D-PSH has yet to be characterized completely due to the lack of sufficiently sensitive and selective methods for the determination of its metabolites.

The primary pathway for D-PSH catabolism is via disulfide formation, e.g. by oxidation (eqns. 1 and 2) and by thiol/disulfide exchange (eqn. 3) [20].

$$2\text{PSH} \xrightarrow{O_2} \text{PSSP} \quad (1)$$

$$\text{PSH} + \text{RSH} \xrightarrow{O_2} \text{PSSR} \quad (2)$$

$$\text{PSH} + \text{RSSR} \rightleftarrows \text{PSSR} + \text{RSH} \quad (3)$$

where RSH can be a small molecule, e.g. cysteine or glutathione, or a protein. With the development of electrochemical detectors with dual gold/mercury (Au/Hg) amalgam electrodes for HPLC, thiols and their disulfides can be determined simultaneously [21–26]. In this paper, we described the results of a study of the use of HPLC–ED for the determination of D-PSH, its symmetrical disulfide (PSSP) and its mixed disulfide with glutathione (PSSG). The electrochemical behavior of the symmetrical and mixed disulfides at the Au/Hg amalgam electrode was characterized, and mobile phase conditions were established for separation of the various thiols and disulfides by reversed-phase ion-pairing HPLC. Procedures were also developed for the preparation of calibration curves for the mixed disulfide which do not require the mixed disulfide in pure form.

EXPERIMENTAL

Chemicals

Reduced and oxidized glutathione (GSH and GSSG, respectively), reduced and oxidized penicillamine (PSH and PSSP) and sodium octyl sulfate (SOS) were obtained from Sigma (St. Louis, MO, U.S.A.). The water used for preparation of mobile phases was twice distilled and then deionized by passage through a mixed bed resin in a Barnstead (Division of Sybron, Boston, MA, U.S.A.) water purification system. Reagent-grade methanol (AnaChemie, Montreal, Canada) was distilled in glass. All samples and solutions, including mobile phases, were filtered through 0.2-μm Nylon 66 membrane filters manufactured by Schleicher and Schuell (distributed by Mandel Scientific, Edmonton, Canada).

Equipment

Chromatography was performed on a Bioanalytical Systems (Purdue Industrial Research Park, West Lafayette, IN, U.S.A.) LC-154 liquid chromatograph equipped with an electrochemical detector with dual Au/Hg amalgam electrodes (Bioanalytical Systems, Model TL-6A) in the series configuration. Bioanalytical Systems Biophase 5 μm particle size octadecyl silane (ODS) and Whatman Partisil 5 μm particle size ODS-3 columns (250 mm $\times$ 4.6 mm I.D.) were used (Whatman, Clifton, NJ, U.S.A.). A 4.0 cm $\times$ 4.6 mm I.D. guard column packed with 10 μm particle size Partisil ODS-3 was inserted between the 20-μl sample loop injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.) and the analytical column. A mobile phase consisting of phosphate buffer was used in all the studies. Determination of the optimum mobile phase composition is described in the Results section. The mobile phase was refluxed under nitrogen at 30°C with a water-filled bubbler attached to the top of the condenser to prevent oxygen from entering the mobile phase. A flow-rate of 1.0 ml/min was used. Nuclear magnetic resonance (NMR) measurements were made at 400 MHz with a Bruker (Billerica, MA, U.S.A.) WH-400 spectrometer operating in the pulse–Fourier transform mode.

Solutions

Stock solutions (generally 0.001–0.01 *M*) of the thiols and disulfides were prepared in deoxygenated pH 3.0 phosphate buffer (0.1 *M*) containing 0.001 *M* Na_2H_2EDTA. To minimize autoxidation of the thiols, the buffer was deoxygenated by refluxing at 40–50°C for about 15 min; the buffer was bubbled with nitrogen during refluxing and while it was cooling. The nitrogen was deoxygenated by bubbling through an oxygen scrubber solution made of acidified ammonium metavanadate [27] and then through water to prevent any contamination by the aerosol from the vanadium solution. Stock solutions were diluted as necessary with buffer solution.

Since the PSSG was not available in pure form, it was prepared by thiol/disulfide exchange [28]:

$$PS^- + GSSG \rightleftarrows PSSG + GS^- \quad (4)$$

$$PS^- + PSSG \rightleftarrows PSSP + GS^- \quad (5)$$

Solutions of PSH and GSSG (0.05–0.1 *M*) were prepared in pH 3.0 phosphate–^2H_2O buffer containing 0.001 *M* Na_2H_2EDTA. A 1-ml volume of each solution was pipetted into a 5-ml volumetric flask and the pH was increased to 11.0 with solid NaOH. After reaction for 20 min, the solution was acidified with H_3PO_4–^2H_2O (50:50, v/v) to pH 3.0 or lower to stop the reaction [28]. The flask was diluted to volume with pH 3.0 phosphate–^2H_2O buffer and a 0.5-ml aliquot placed in an NMR tube for analysis by ^1H NMR. The remainder of the solution was used for preparation of calibration standards and for spiking bi-

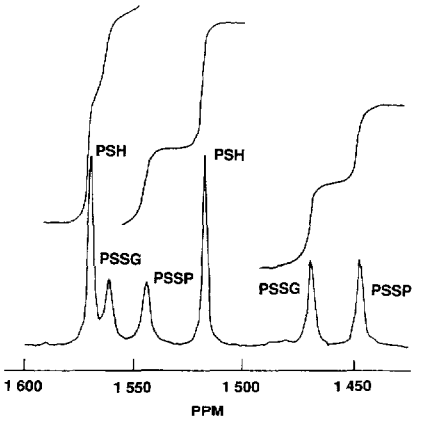

Fig. 1. A portion of the 400-MHz ^{1}H NMR spectrum of a solution prepared by reaction of 0.127 *M* PSH with 0.106 *M* GSSG using the reaction conditions described in the text. The resonances are for the methyl protons of penicillamine in the forms indicated.

ological fluids. Since the rate of the reaction of PSH with PSSG is low [28], little or no PSSP was generally formed by eqn. 4, in which case PSSP was added from a separate stock solution when spiking biological fluids.

The concentrations of the thiols and disulfides in the thiol/disulfide exchange reaction mixture were determined by ^{1}H NMR. Fig. 1 shows a portion of a typical spectrum for a reaction mixture containing PSH, GSH, PSSG, GSSG and PSSP, together with the integral traces as determined by the spectrometer software. The chemical shifts are relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DDS). The resonances in Fig. 1 are for the methyl protons of PSH, PSSP and PSSG; two methyl resonances are observed for each molecule because of the asymmetric center at the α-carbon [28]. The fraction of the total PSH in each form was determined from the integrated intensity for the methyl resonances for that form and the total integrated intensity. Using these fractions and the initial PSH concentration, the concentrations of PSH, PSSP and PSSG in the reaction mixture were calculated. Finally, the concentrations of GSH and GSSG were calculated from these concentrations and the appropriate mass balance relationships.

Sample preparation

Venous blood from healthy human volunteers was drawn into vacutainers (Becton, Dickinson, Rutherford, NJ, U.S.A.) which contained K_3HEDTA. Plasma was separated from red blood cells by centrifugation with a Damon/IEC Division (Needham Heights, MA, U.S.A.) Model HNS centrifuge at 1000 *g* at 25 °C for 10 min. Plasma proteins were precipitated by addition of an equal volume of cold 5% (w/v) trichloroacetic acid (TCA) solution to plasma and centrifugation at 1000 *g* at 25 °C for 10 min after waiting 5 min at 0 °C for

complete precipitation of protein. The supernatant was filtered through a 0.2-μm membrane filter and then spiked with the thiol–disulfide standard solution.

Red blood cells were isolated from whole blood by centrifugation at 1000 *g* at 4°C for 10 min followed by removal of plasma and white cells by aspiration. The red blood cells were washed four times with an N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid (HEPES, Sigma) buffered saline solution containing 0.15 *M* NaCl, 2% (w/v) glucose and 0.015 *M* HEPES at pH 7.5. After washing, the cells were diluted about 20:80 (v/v) in saline solution and the hematocrit was measured. Typically, 0.3 ml of the cell suspension was then pipetted into a 1.5-ml centrifuge tube, 0.3 ml of an isotonic solution containing D-PSH (1–10 m*M*) was added, and the cells were incubated for 30–90 min in the D-PSH solution at 37°C. After incubation, the cells were put on ice to stop any further transport of D-PSH [29], centrifuged for 10 s at 15 600 *g* and 25°C with an Eppendorf Model 5142 centrifuge (distributed by Brinkmann Instruments, Westbury, NY, U.S.A.), and the supernatant was removed. The cells were washed four times with 0.106 *M* $MgCl_2$, lysed with 0.5% (v/v) Triton X-100 solution, and then cold 5% (v/v) TCA solution was added to precipitate the protein. After centrifugation for 10 s at 15 600 *g* and 25°C, the supernatant was filtered through 0.2-μm membrane filters, diluted as necessary, and then analyzed by HPLC.

Urine samples were prepared by pipetting 2 ml into a 25-ml volumetric flask and diluting to volume with cold 10% (w/v) TCA solution. The solution was then transferred to a plastic centrifuge tube, kept on ice for 5–10 min for complete protein precipitation and centrifuged at 1000 *g* and 25°C for 10 min. The supernatant was filtered through a 0.2-μm membrane filter and spiked with thiol–disulfide mixtures just prior to analysis.

RESULTS

Hydrodynamic voltammograms

The electrochemical detector used in this work had two Au/Hg amalgam working electrodes in series. Chromatograms were obtained by measuring the current through the downstream electrode. The downstream electrode was set to a potential at which thiols facilitate oxidation of the Hg electrode

$$2RSH + Hg \rightarrow Hg(SR)_2 + 2e^- + 2H^+ \quad (6)$$

The upstream electrode was operated at a negative potential to convert disulfides to thiols for detection at the downstream electrode.

$$RSSR + 2e^- + 2H^+ \rightarrow 2RSH \quad (7)$$

To determine the optimum potential for the downstream electrode, hydrodynamic voltammograms were measured for PSH and GSH. The procedure involved measuring chromatographic peak currents for injected thiols as a

function of the applied electrode potential. The oxidative plateaus, i.e. the potential ranges over which the peak current due to the reaction in eqn. 6 varies little with change in potential, are −0.22 to +0.24 V (vs. the Ag/AgCl reference electrode) for PSH and −0.10 to +0.20 V for GSH with a pH 3.0 mobile phase (0.1 *M* phosphate). The downstream electrode was set to +0.150 V vs. the Ag/AgCl electrode in all of the following measurements, so that the peak current would be independent of small fluctuations in electrode potential.

To determine the appropriate potential for the upstream electrode, hydrodynamic voltammograms were measured for PSSP, PSSG and GSSG by measuring chromatographic peak currents vs. the potential of the upstream electrode with the downstream electrode set to +0.15 V vs. the Ag/AgCl electrode. The ratio of the peak current at a given potential to the maximum peak current on the reduction plateau is plotted vs. the potential of the upstream electrode in Fig. 2 for GSSG, PSSG and PSSP. It is interesting to note that the reduction wave for PSSP is shifted to negative potential by ~0.23 V relative to that for GSSG, while the reduction wave for PSSG is located between those for PSSP and GSSG. Presumably a more negative potential is required for reduction of PSSP because of steric effects associated with the four methyl groups, as has

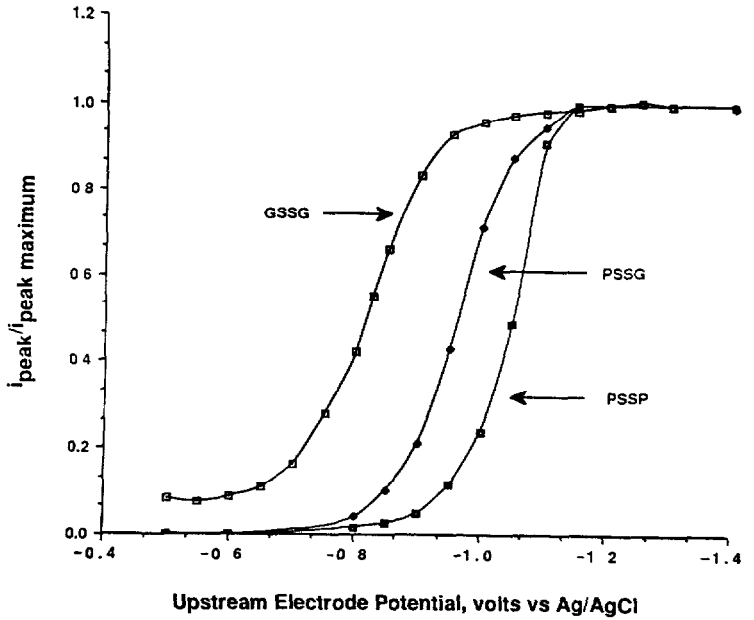

Fig. 2. Hydrodynamic voltammograms for GSSG, PSSG and PSSP in pH 3.0 phosphate buffer. The downstream electrode was set to a potential of +0.150 V vs. the Ag/AgCl electrode while the potential of the upstream electrode was varied. The ordinate is the ratio of peak current at the given potential to the maximum peak current response with the upstream electrode at −1.30 V.

been observed previously for the chemical reduction of PSSP by thiol/disulfide exchange [28]. The upstream electrode should be set to a potential 50–100 mV more negative than the onset of the reduction plateau so that the measured peak currents will not be affected by small changes in the actual electrode potential. Thus, for PSSP, the upstream electrode should be set at −1.20 V vs. the Ag/AgCl electrode. However, rapid deterioration of the surface of the upstream electrode was observed at this potential. A compromise between detector sensitivity and electrode lifetime was made by setting the upstream electrode to −1.100 V.

Others [21,30] have reported fast degradation of the Au/Hg electrode at high potentials and therefore use an upstream potential of −1.00 V [21]. However, the hydrodynamic voltammogram for PSSP indicates that, at this electrode potential, peak currents will only be about 25% of the maximum current obtained with the upstream electrode set to a potential on the reduction plateau. We have found that use of a potential of −1.100 V with fresh and fully deoxygenated mobile phase produces a higher response for PSSP without excessive electrode deterioration. Under these conditions, about 88% of the maximum plateau current was achieved.

The average lifetime of the upstream electrode was about one week with continual use, although this was reduced somewhat when the mobile phase contained a high (i.e. 100 mg/l) concentration of SOS. With SOS present, the upstream electrode was the first to lose its mercury. It could, however, be re-amalgamated separately by rinsing with 98% (v/v) ethanol, air-drying and then placing a single drop of mercury on the electrode. After several minutes, the excess mercury was removed, being careful to prevent the mercury from coming in contact with the downstream electrode. Re-amalgamation of only the upstream electrode, when the downstream electrode was still satisfactory, minimized the time required for the downstream electrode to stabilize to a constant background current. Because the upstream electrode was used only to reduce disulfide, its current was not measured and thus, equilibration of the upstream electrode was not necessary.

Chromatographic conditions

To identify mobile phase conditions for optimum separation of the various thiol–disulfide mixtures, mobile phase parameters were systematically varied. First, capacity factors were measured for PSH, GSH, PSSP and GSSG as the pH of a 0.1 *M* phosphate mobile phase was varied over the pH range 2.5–5.5. It was assumed that PSSG would elute at some time intermediate between the elution times for PSSP and GSSG since it has a structure intermediate between these two compounds. The pH range 2.5–5.5 was used since it is within the working range of the reversed-phase C_{18} column and because the rate of thiol oxidation decreases significantly below pH 5.5 [28]. In general, the capacity factors either decreased (PSH, GSH, GSSG) or remained relatively

constant (PSSP) as the pH was increased. For samples containing PSH, GSH, GSSG and PSSP, the GSH and PSH are the most difficult to separate. Better separation was achieved at pH 2.5 than at higher pH; however, tailing was also greater at pH 2.5. A pH of 3.0 was selected; at this pH, the separation factor for PSH and GSH is less than at pH 2.5 (1.21 vs. 1.30); however, resolution is better (1.56 vs. 1.20) because the peaks are narrower. The chromatogram for a mixture of GSH, PSH, PSSP, PSSG and GSSG is shown in Fig. 3. As expected, the retention time for PSSG is intermediate between those of PSSP and GSSG.

To improve the separation of GSH and PSH further, the effect of ion-pairing reagent (SOS) and organic modifier (methanol) on capacity factors was studied. Studies indicated that the combined effects of the ion-pairing reagent and the methanol are to increase the separation of GSH and PSH while preventing the retention times for the disulfides from becoming too long. From the results of these studies, a pH 3.0 mobile phase containing 0.80 m*M* SOS and 3% (v/v) methanol was selected. A chromatogram of an aqueous solution containing GSH, PSH, GSSG, PSSG and PSSP and spiked with cysteine (CSH) is shown in Fig. 4. The chromatogram shows good separation of GSH and PSH, and the retention times for the dilsulfides are reasonable. It is also interesting to note that the elution order for GSSG and PSSP is opposite that obtained with the pH 3.0 mobile phase; the effect of an increasing concentration of SOS is to cause the capacity factor for PSSP to increase more rapidly than that for GSSG while the effect of an increasing concentration of methanol is to cause the capacity factor for GSSG to decrease more rapidly than that for PSSP.

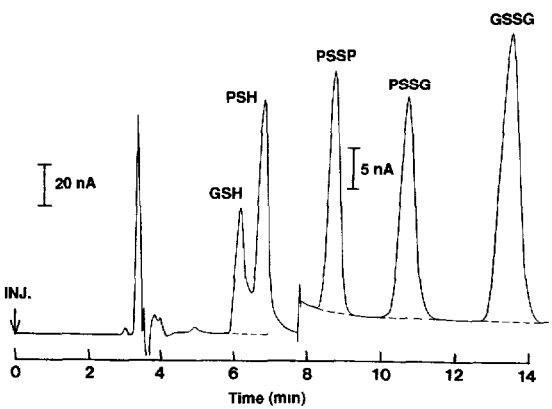

Fig. 3. Chromatogram of an aqueous solution containing PSH, GSH, PSSP, PSSG and GSSG. The mobile phase was pH 3.0 phosphate buffer. The upstream and downstream electrodes were set to −1.100 and +0.150 V vs. Ag/AgCl, respectively. At 7.75 min, the sensitivity was changed from 200 to 50 nA full scale.

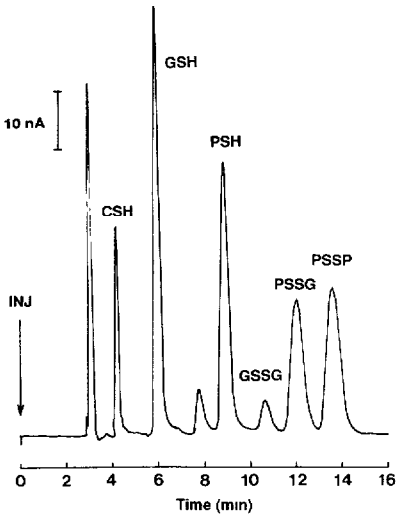

Fig. 4. Chromatogram of an aqueous solution containing PSH, GSH, PSSP, PSSG, GSSG and cysteine (CSH). The mobile phase was pH 3.0 phosphate buffer plus 0.80 m*M* SOS and 3% (v/v) methanol. The peak at ~7.8 min has not been identified

Calibrations

The response of the detector was calibrated by measuring peak currents for standard solutions of thiols and disulfides. To characterize the reproducibility of peak-current measurements, peak currents were measured for replicate injections of 25 μM GSH and of 275 μM GSSG. Average peak currents ($\pm$S.D.) of 69.7 ± 0.6 and 115 ± 1 nA were obtained for GSH and GSSG from twelve and seven injections, respectively.

To characterize the stability of detector response with time and to evaluate the lifetime of the electrodes, the electrodes were prepared and mobile phase was passed through the detector (upstream electrode set to -1.10 V, downstream electrode $+0.15$ V) for two days before use. Then calibration standards were chromatographed repeatedly over a four-day period. The electrodes were not polished or resurfaced with mercury during this period. However, at the end of each day during the test period, the detector cell was opened to check for any visible signs of electrode deterioration.

The slopes of the lines obtained by linear least-squares fits of the calibration data are listed in Table I. The sensitivity of the detector, as indicated by the slope, was found to decrease with time; however, the detector response vs. concentration was linear up to the fourth day. On the fourth day, the response was non-linear for PSSP and GSSG. Upon inspection, the upstream electrode was found to have a gold tinge indicating a loss of Hg from the surface. The results in Table I indicate the need for frequent calibration of the detector and also that highest sensitivity is obtained with a freshly prepared electrode surface.

TABLE I

SLOPES OF CALIBRATION CURVES FOR GSH, PSH AND GSSG OVER A FOUR-DAY PERIOD

Mobile phase: 0.1 *M* phosphate containing 7.5 mg/l SOS (pH 3.0).

Compound	Concentration range (μM)	Slope (nA·l/mol)			
		Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=4$)	Day 4 ($n=4$)
GSH	0–109	$1.718 \cdot 10^6$	$1.387 \cdot 10^6$	$0.924 \cdot 10^6$	$0.562 \cdot 10^6$
PSH	0–122	$1.804 \cdot 10^6$	$1.222 \cdot 10^6$	$0.777 \cdot 10^6$	$0.401 \cdot 10^6$
GSSG	0–376	$0.334 \cdot 10^6$	$0.286 \cdot 10^6$	$0.223 \cdot 10^6$	—[a]
PSSP	0–640	$0.063 \cdot 10^6$	$0.015 \cdot 10^6$	$0.027 \cdot 10^6$	—[a]

[a]Response not linear.

TABLE II

CALIBRATION DATA FOR PSH, GSH, PSSP, GSSG AND PSSG

Six concentrations were run for each compound, and each concentration was run in duplicate. Peak current (i_p) = slope (concentration) + intercept where i_p is in nA and concentration is in mol/l.

Compound	Slope (mean ± S.D.) (nA·l/mol)	Intercept (mean ± S.D.) (nA)	Correlation coefficient
PSH	$1.216\ (\pm 0.027) \cdot 10^6$	-0.186 ± 0.313	0.9990
GSH	$1.432\ (\pm 0.020) \cdot 10^6$	-0.378 ± 0.176	0.9996
PSSP	$0.0413\ (\pm 0.001) \cdot 10^6$	-0.767 ± 0.637	0.9993
GSSG	$0.392\ (\pm 0.001) \cdot 10^6$	0.042 ± 0.033	1.0000
PSSG	$0.595\ (\pm 0.004) \cdot 10^6$	0.019 ± 0.113	0.9999

Calibration plots of peak current vs. concentration for GSH, PSH, PSSP, PSSG and GSSG obtained using freshly prepared electrodes showed good linearity; however, those for GSH and PSH had a negative *y*-axis intercept. It was found that addition of cysteine, which is less retained than PSH and GSH, resulted in calibration curves with an approximately zero *y*-axis intercept. Apparently, there is some loss of GSH and PSH by adsorption onto the mercury of the upstream electrode, which is minimized by adsorption of the less retained cysteine (Fig. 4). Calibration curves were prepared by measuring chromatographic peak height vs. concentration for the following concentration ranges: GSH, 0–15 μM; PSH, 0–25 μM; PSSG, 0–60 μM; GSSG, 0–50 μM; PSSP, 0–1500 μM. The detector response increased linearly with concentration over these concentration ranges; the slopes and intercepts of the calibration plots are listed in Table II. The mobile phase was pH 3.0 phosphate buffer

which contained 0.80 m*M* SOS and 3% (v/v) methanol. These data indicate that the sensitivity of the detector is somewhat lower for PSSP than for the other thiols and disulfides. Detection limits at a signal-to-noise ratio of 2.0 were found to be 0.3 μM for GSH and PSSG and 0.1, 0.4 and 30 μM for PSH, GSSG and PSSP, respectively, for 20 μl of injected solution.

PSH, GSH, PSSP, PSSG and GSSG in human plasma

Calibration curves and plasma were prepared as described above. The plasma supernatant was spiked with a standard mixture of PSH, GSH, PSSP, PSSG and GSSG; Fig. 5 shows a typical chromatogram for a spiked plasma sample. The peaks preceding the GSH peak are due to electroactive impurities in the TCA used to precipitate plasma proteins and to the added cysteine. Replicate analyses of eight different spiked samples were performed in a recovery study. Concentration ranges for the spiked samples were: GSH, 6–23 μM; PSH, 7–40 μM; GSSG, 6–50 μM; PSSG, 6–23 μM; PSSP, 71–745 μM. The mean recovery ($\pm$S.D.) were: GSH, 96 $\pm$ 4%; PSH, 86 $\pm$ 12%; GSSG, 99 $\pm$ 4%; PSSG, 99 $\pm$ 4%; PSSP, 139 $\pm$ 27%. Good recoveries were obtained for all but PSH and PSSP; the low recovery for PSH and the high recovery for PSSP suggest that some PSH was oxidized in the spiked plasma. However, considering the different concentration ranges for PSH and PSSP, this can account for only a small fraction of the high recovery for PSSP. Quantitative recoveries were observed when buffer was spiked with the same mixtures, suggesting an effect from the

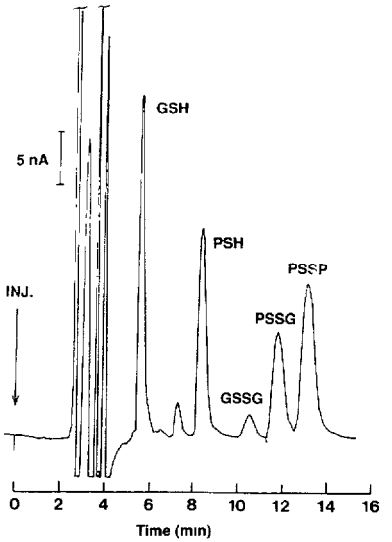

Fig. 5. Typical chromatogram of a plasma sample spiked with GSH, PSH, GSSG, PSSG and PSSP. The peaks preceding the GSH peak are due to cysteine and to electroactive impurities in the TCA.

TABLE III

PSH, GSH, GSSG AND PSSG CONCENTRATIONS IN HUMAN ERYTHROCYTES AFTER INCUBATION IN ISOTONIC 10 m*M* PSH SOLUTION

Incubation time (min)	Concentration (m*M*)			
	GSH	PSH	GSSG	PSSG
30	1.83	6.75	0.24	0.17
60	1.95	11.05	0.24	0.17
90	1.77	12.19	0.10	0.19

plasma, although no extraneous peaks were observed in chromatograms for supernatant which had not been spiked.

PSH, GSH, PSSP, PSSG and GSSG in human urine

Supernatant from urine samples prepared as described above were spiked with standard mixtures of PSH, GSH, PSSP, PSSG and GSSG to determine recoveries. Six recovery studies were performed in the concentrations ranges 9–42 μM for PSH, 8–39 μM for GSH, 242–599 μM for PSSP, 3–37 μM for PSSG and 2–30 μM for GSSG. Mean ($\pm$S.D.) recoveries were: PSH, $95 \pm 7\%$; GSH, $99 \pm 4\%$; PSSP, $119 \pm 25\%$; PSSG, $94 \pm 2\%$; GSSG, $90 \pm 7\%$. As was observed in the plasma recovery studies, the PSSP recovery is high.

PSH, GSH, PSSG, PSSP and GSSG in human erythrocytes

Human erythrocytes were incubated in an isotonic solution which contained 10 m*M* D-PSH for periods of 30, 60 and 90 min, after which the cells were separated from the incubation medium, and protein-free supernatants were prepared as described above. The concentrations of GSH, PSH, GSSG and PSSG in the erythrocytes as determined by HPLC analysis of the supernatants are presented in Table III. The concentration of PSSP was below the detection limit of the electrochemical detector; using the detection limit presented above for PSSP and accounting for dilution in the preparation of the supernatant, the concentration of PSSP in the intact erythrocytes was estimated to be less than 1.5 m*M*. The variation in total glutathione concentration is probably due in part to uncertainty in the hematocrit and to loss of cells during the washing steps.

DISCUSSION

The results of this study provide a method for the simultaneous determination by HPLC–ED of PSH, PSSP and PSSG in biological fluids. Several procedures for the determination of reduced PSH in aqueous solution and in bio-

logical fluids and for the determination of PSSP in aqueous solution by HPLC–ED have been reported previously [14–19,22]. Also, the determination of total non-protein oxidized PSH (i.e. PSSP plus PSH mixed disulfides with small biological molecules) in urine, plasma and erythrocytes by HPLC–ED has been reported [14]. The procedure involved determination of reduced PSH in the deproteinized sample, followed by electrolytic reduction of the non-protein disulfides in another aliquot and then determination of the total PSH. Thus no information was obtained about the chemical forms of the oxidized PSH.

The methodology developed in the present study should be particularly useful for the determination of PSH and its oxidized forms in erythrocytes, e.g. in studies of the transport of PSH across the erythrocyte membrane [31] as well as in studies of PSH pharmacokinetics since GSH is the major non-protein thiol in erythrocytes [32]. The results in Table III indicate the formation of PSSG in erythrocytes incubated in penicillamine solution; however, the majority of the intracellular PSH is in the reduced form. This was also found to be the case in erythrocytes from patients on PSH therapy for rheumatoid arthritis [14]. Unfortunately, the detection limit for PSSP is not low enough to determine the PSSP, if any, in the erythrocytes incubated in PSH solution. It is interesting to note that, although the electrochemical and chromatographic properties of PSSG are intermediate between those of PSSP and GSSG, the detection limit for PSSG is similar to that for GSSG.

Since PSSG is not readily available in pure form, it was prepared in the present study by a thiol/disulfide exchange reaction (eqn. 4) and the resulting solution was standardized by ^{1}H NMR. This method is convenient and can have a precision and accuracy of $\pm 1\%$ if careful attention is paid to the NMR measurement conditions. In particular, the ^{1}H NMR spectra must be measured under quantitative conditions. Fortunately, the methyl resonances of PSH and its disulfides are in a part of the spectrum which is free from other resonances. This region is also free of resonances for most other biological thiols, so that this approach can be generally used for the preparation of standard solutions of mixed disulfides of PSH and biological thiols when pure samples of the mixed disulfides are not available.

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