Biochemical Pharmacology, Vol. 36, No. 8, pp. 1197–1201, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

REVERSIBILITY OF DISULFIDE FORMATION

COMPARISON OF CHEMICAL AND ENZYME-MEDIATED REDUCTION OF PENICILLAMINE AND CAPTOPRIL DISULFIDES

OLAF H. DRUMMER, L. ROUTLEY and N. CHRISTOPHIDIS
University of Melbourne, Clinical Pharmacology and Therapeutics Unit, Austin Hospital, Heidelberg, 3084, Australia

(*Received* 3 *July* 1986; *accepted* 21 *October* 1986)

Abstract—The reduction of penicillamine disulfide by reductants in aqueous solutions has been studied and compared with that for captopril disulfide. Whereas near quantitative reduction for captopril disulfide was achieved with tributyl phosphine (200 mM), no detectable penicillamine was formed from penicillamine disulfide. Thiol reductants (25 mM) were, however, partially able to reduce penicillamine disulfide with the most effective agent being glutathione (15% reduction) following by dithioerythritol (8%) and cysteine (5.1%). The reduction of penicillamine-cysteine disulfide by glutathione was 6-fold higher than for penicillamine disulfide.

Kinetic analysis showed that the initial rate of reduction and equilibrium constant for the reduction of penicillamine disulfides by glutathione were 267- and 875-fold less than for captopril disulfide at pH 7.4.

Biotransformation studies in the cytosol fraction of rat blood cells demonstrated that whereas 48% of the reduction of captopril disulfide was enzyme-mediated only 19% of the penicillamine formed was enzyme-mediated for penicillamine disulfide. Accumulation of disulfides of penicillamine in patients taking penicillamine may therefore be a problem during chronic therapy.

D-Penicillamine (P), a thiol amino acid is known readily to form disulfide conjugates with other thiols such as cysteine, glutathione and proteins in a similar manner to captopril (C) [1–3]. The pharmacological actions of P probably primarily reside in the reduced form. Recent work on CP shows that the extent of disulfide formation *in vivo* and the ability of these disulfides to be reduced back to C play an important role in determining the pharmacokinetics and the duration of action of C [4].

In contrast to captopril disulfides the reduction of penicillamine disulfides is, however, quite difficult and is presumably related to steric hinderance of the B-B-dimethyl group adjacent to the sulfhydryl moiety [5]. However, reduction particularly of the high molecular weight forms is essential for complete clearance of P *in vivo* since these forms are not excreted by the kidneys [6]. Knowledge of the mechanism and rate of this reduction process is therefore important in understanding the disposition of P. However, there is little known about the reduction of penicillamine disulfides probably because of the difficulty in adequately measuring P. We have recently published a specific and accurate electrochemical HPLC method for P [7] and we have used this method to examine the rate and extent of reduction of penicillamine disulfides by thiol and non-thiol reductants including the endogenous thiol glutathione and have compared this to captopril disulfide. We also examine the involvement of glutathione and oxidoreductase enzymes for these disulfides in the reduction process *in vivo* using rat red blood cell cytosol preparations.

MATERIALS AND METHODS

Reagents and chemicals

P (gift from Eli Lilly, Aust.), *N*-acetylcysteine (Sigma; St. Louis, MO), C (gift from E. R. Squibb Inc., Aust.) were prepared daily as 1 mg/ml stock solutions in 1 mg/ml EDTA solution. Penicillamine disulfide (PD) (Sigma, St. Louis, MO) and captopril disulfide (CD) (gift from E. R. Squibb Inc., Aust.) dimers were prepared daily as 10 mg/ml and 3 mg/ml solutions respectively in 25 mM sodium hydroxide. Penicillamine-cysteine disulfide was prepared from P and cysteine (Sigma, St. Louis, MO) according to Crawhall *et al.* [8].

Dithioerythritol (Serva, Heidelberg, F.R.G.), glutathione (Calbiochem, San Diego, CA), tributylphosphine (Aldrich, Milwaukee, WS), NADPH and NADH (Boehringer-Mannheim, F.R.G.) were used as obtained. All other reagents were analytical reagent grade.

Reduction experiments

Penicillamine disulfides. Experiments involving reduction of penicillamine disulfide dimer and penicillamine-cysteine disulfide were carried out in a total volume of 500 μl in 0.1 M potassium phosphate buffer (pH ranges: 6.0, 7.4, 9.0, 10.0) in disposable glass test tubes. Incubations were all conducted at 37° for varying times in the presence of various reductants. When thiols were used as reductants stock solutions (10 or 100 mg/ml) were made in distilled water. When tributyl phosphine was used it

was added directly to the incubation tube (carried out in a fume cupboard). In some experiments oxygen was excluded by purging all solutions with nitrogen both before and during the reduction experiments.

Reactions were stopped by the addition of 250 μl of 1 M perchloric acid in 1 mg/ml EDTA solution. Standards containing known amounts of P were prepared in the same manner, except perchloric acid was immediately added. Internal standard (5 μg of *N*-acetylcysteine) was added to all tubes after the addition of perchloric acid. The pH of all tubes were then adjusted to 2.5 with 0.8 M pH 9.0 potassium phosphate buffer (usually 75–100 μl). Five microlitres of supernatant was injected into the HPLC after a brief 5 min centrifugation (100 *g*.max). The HPLC conditions were as described by Drummer *et al.* [7].

Captopril disulfide. When CD was used the reduction conditions were identical to that for penicillamine disulfides except that the addition of *N*-acetylcysteine, as internal standard, was omitted.

The formed C was analysed by liquid chromatography using the same Gold/Mercury electrochemical cell as for P. The Au/Hg cell was maintained at +0.13 V versus a Ag/AgCl reference electrode. The mobile phase consisted of 40% methanol in 0.1 M chloroacetic acid which had previously been refluxed under nitrogen for 3 hr prior to use. Chromatography was performed on a 15 cm Whatman partisil-ODS-3 column (3.8 mm i.d.) packed with 3 μm particle size C18-bonded phase protected by a 2 cm guard-column dry-packed with C18-bonded pellicular packing (Waters Associates). Flow rate was maintained at 1.0 ml/min. Under these conditions C gave a sharp symmetrical peak at 6.0 min. No interference from other thiols was noted.

Preparation of tissue cytosol fractions

Red blood cell cytosol preparation of Sprague–Dawley rats (weight 200–260 g) was prepared by centrifuging blood collected in heparin tubes at 1000 *g*.max for 10 min at room temperature and resuspending the red blood cells with an equal volume of normal saline. Following centrifugation (1000 *g*.max, 10 min) the cells were resuspended and centrifuged once more with normal saline. The washed cells were then treated with an equal volume of 10 mM potassium phosphate buffer (pH 7.4), placed on ice, and after 30 min, homogenized in a glass homogenizer (10 strokes). All samples were then centrifuged at 10,000 *g*.max for 15 min. The supernatant fraction was then used for the experiments.

Enzyme studies

Cytosolic fractions (400 μl) of rat red blood cells were added to tubes containing 100 μl of 0.1 M potassium phosphate buffer (pH 7.4) containing (final concentrations) PD (1 mM) and either NADH or NADPH (3 mM), and where indicated GSH (2.5 mM). Incubations proceeded at 37° for specified periods of time and were stopped by the addition of 250 μl perchloric acid (1 M). Analysis of formed P or C was as described earlier. Heat-treated samples were prepared by incubating cytosol fractions in a 80° water bath for 5 min. After cooling (4°) and centrifugation (1000 *g*.max, 10 min) the supernatant fraction was used for enzyme studies.

RESULTS

(i) *Effect of chemical reductants*

The *in vitro* reduction of disulfides to the thiol was determined by the appearance of P in a pH 8.0 phosphate buffer over a 2 hr period, using a concentration of PD of 1 mM. Thiol reductants in a 2.5 molar excess such as dithioerythritol, cysteine and glutathione only weakly reduced PD to P (Table 1). Under these conditions glutathione was the most effective giving 1.85% reduction (% of initial PD) whereas dithioerythitol and cysteine gave only 0.49% and 0.27% reduction respectively. Tributylphosphine at 20 mM and 200 mM was, however, totally ineffective in reducing PD to P but quantitatively reduced CD to C (Table 1). The reduction of CD was best effected by dithioerythritol. At 2.5 mM dithioerythritol gave a quantitative reduction whilst conversion to C by 2.5 mM cysteine and glutathione were 29% and 47% respectively. At 25 mM both these thiols gave quantitative reduction of CD. In the absence of reductant no significant formation of either P or C was detected.

(ii) *pH dependence of penicillamine disulfide reduction*

At pH 8.0 glutathione gave a time-dependent conversion of PD to P (Fig. 1). At 4 hr, the last time point studied, 4.2% of PD was reduced. The rate of reduction was, however, increased at pH 9.0 with 6.0% reduced at 4 hr (Fig. 1), however, the reduction was not linear after 2 hr. Increasing the pH to 10 substantially reduced both the rate and extent of reduction (2.1% at 4 hr) as well as exhibiting a plateau effect at 2 hr beyond which no further PD was reduced indicating that equilibrium had been reached at 2 hr. At pH 7.4 the rate of reduction was significantly less than at pH 8.0 ($P < 0.05$; Student's *t*-test). The 4 hr conversion to P was 1.6% at pH 7.4. No perceptible reduction of PD occurred at pH 6.0 (<0.2% at both 2 hr and 4 hr).

(iii) *Effect of glutathione concentration on the reduction of penicillamine disulfide*

Glutathione showed a concentration dependent reduction of PD to P up to a glutathione concentration of 50 mM (Fig. 2). At 50 mM (using a 1 mM PD concentration throughout) the extent of conversion to penicillamine had reached 23 ± 5% ($N = 4$). However, beyond 10 mM glutathione the reduction was no longer linear. Concentrations of glutathione higher than 50 mM could not be used because glutathione began to interfere with the HPLC analysis of P.

(iv) *Reduction of penicillamine–cysteine disulfide by glutathione*

The reduction of another metabolite of P, penicillamine–cysteine mixed disulfide (at 1 mM) to P by 2.5 mM glutathione was maximal at 30 min giving 8.3 ± 3.6%, which was 6-fold higher than for PD.

Table 1. Reduction of penicillamine and captopril disulfides by various reductants

Reductant	Concentration of reductant (mM)	% Conversion to penicillamine	% Conversion to captopril
Cysteine	2.5	0.27 ± 0.06	29 ± 6
	25	5.1 ± 1.1	100 ± 4
Glutathione	2.5	1.8 ± 0.3	47 ± 5
	25	15 ± 3	101 ± 4
Dithioerythritol	2.5	0.49 ± 0.08	117 ± 8
	25	8 ± 2	90 ± 6
Tributylphosphine	20	0.0	70 ± 8
	200	0.0	95 ± 4

Disulfide (1 mM) incubated in the presence of reductant for 2 hr at 37° in pH 8.0 potassium phosphate buffer. Mean of three to four experiments ± SEM.

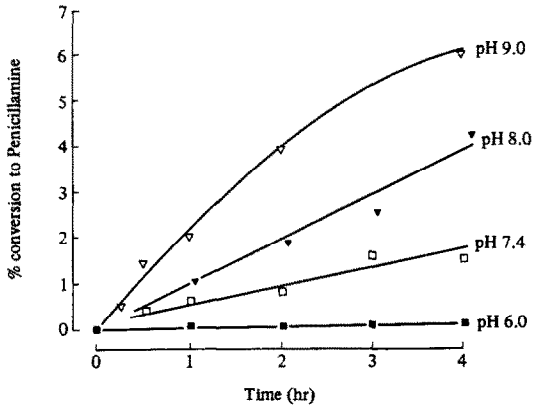

Fig. 1. Percent conversion of penicillamine disulfide dimer (initial concentration 1 mM) to pencillamine *in vitro* at 37° by 2.5 mM (reduced) glutathione in 0.1 M potassium phosphate at (a) pH 6.0, ■; (b) pH 7.4, □; (c) pH 8.0, ▼; and (d) pH 9.0, ▽. Means of 5 separate experiments assayed in duplicate. Error bars not shown for clarity.

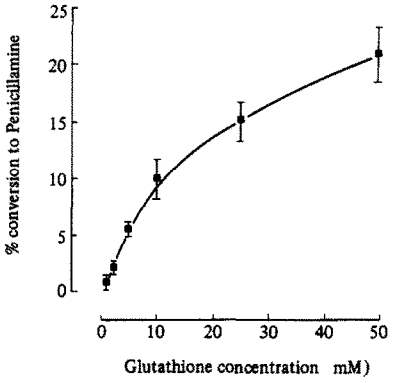

Fig. 2. Time course for the conversion of penicillamine disulfide dimer to penicillamine *in vitro* by increasing concentrations of glutathione at 37° in 0.1 M pH 9.0 potassium phosphate. Means of 3 separate experiments assayed in duplicate.

At 2 hr the conversion of penicillamine cysteine disulfide was less than at 30 min (5.8 ± 2.1%) but this was still greater than that obtained for penicillamine disulfide dimer (3.9% at 2 hr) indicating that equilibrium for this mixed disulfide had been reached much earlier than for PD.

(v) *Rates of reduction and equilibrium constant*

The rate of reduction of CD by glutathione using 1 mM concentrations of reactants was much greater than for the reduction of PD by glutathione. Reduction curves for CD at pH 7.4 and pH 9.0 are shown in Fig. 3. Similar curves for PD have been shown in Fig. 2. The initial rates of reaction for CD at pH 7.4 and pH 9.0 were 16 ± 3 and 43 ± 6 μmol/l/min, respectively. In contrast the initial rates of reaction of PD (using 1 mM concentrations of both PD and glutathione) at pH 7.4 and pH 9.0 were 0.060 ± 0.025 and 0.34 ± 0.09 μmol/l/min, respectively.

At pH 7.4 which is the physiological pH equilibrium was obtained only very slowly for PD occurring at 5 hr whereas for CD equilibrium was obtained at 20 min (Fig. 3). The equilibrium constants for the following reactions were calculated at pH 7.4:

$$PD + GSH \rightleftharpoons P + P\text{-}GSH \quad (1)$$

$$CD + GSH \rightleftharpoons C + C\text{-}GSH \quad (2)$$

where GSH = glutathione, P–GSH = penicillamine–glutathione mixed disulfide, C–GSH = captopril–glutathione mixed disulfide.

Assuming no side reactions were occurring the equilibrium constants for PD and CD were $4.15 \pm 1.1 \times 10^{-5}$ and $3.63 \pm 0.52 \times 10^{-2}$ respectively. Kinetic analysis of the reduction data indicate first order kinetics with respect to both the disulfide and for glutathione.

Although oxygen was not excluded in all the reductions described to date, a further series of experiments were conducted for the reduction of PD (1 mM) by glutathione (1 mM) using oxygen-free reagents and oxygen-free phosphate buffer (pH 7.4) at 37° (see Methods). In these experiments the initial rate of reduction of PD was 0.080 ± 0.016 μmol/l/min and the equilibrium constant was $4.0 \pm 0.7 \times 10^{-5}$. Neither of these values was significantly different to those parameters in which the presence of oxygen was not specifically excluded.

(vi) *Biotransformation in the cytosol fraction of rat red blood cells*

Incubation of both PD and CD (1 mM) with cytosol fractions of lysed rat blood cells resulted in the formation of P and C respectively (Table 2). Both these reactions were, within the limits of exper-

Table 2. Percent formation of penicillamine or captopril with cytosol fraction of rat red blood cells after incubation of the corresponding disulfide dimer in the presence of 3 mM NADH. Results are expressed as mean ± SEM (N = 3–4)

Time (min)	15	30	60
Penicillamine disulfide			
Cytosol fraction	0.26 ± 0.02	0.39 ± 0.05	0.45 ± 0.05
Denatured cytosol	0.22 ± 0.02	0.29 ± 0.01	0.37 ± 0.03
% Enzyme mediated activity	15 ± 8	24 ± 13	17 ± 5
Captopril disulfide			
Cytosol fraction	2.98 ± 0.31	5.02 ± 2.64	8.85 ± 1.79
Denatured cytosol	1.87 ± 0.29	2.50 ± 0.16	3.89 ± 0.50
% Enzyme mediated activity	51 ± 8	47 ± 10	47 ± 5

imental error, linear with respect to enzyme concentration (determined by dilution of the cytosol preparations with buffer). For PD it was, however, difficult to assess the formation of P below enzyme concentrations one-half of that shown in Table 2 due to the small amounts of P produced. The percent reduction of PD in the presence of NADH (3 mM) in the incubation mix was 0.26 ± 0.02 at 15 min. For reduction of PD the reaction was linear only up to 30 min (Table 2). The enzyme-mediated component of this reduction was evaluated by substituting active enzyme with a heat-denatured (80°) cytosol preparation. The average percent enzyme-mediated activity over 60 min was 19 ± 6%.

For CD the percent reduction to C in the presence of NADH (3 mM) over 15 min was 3.0 ± 0.3% (N = 4). The reduction of CD in the experiments was, within the limits of experimental error, linear up to 60 min incubation time (Table 2). Over 1 hr the average enzyme-mediated contribution to the reduction of CD to C was 48 ± 7% which was significantly different to that obtained for PD ($P < 0.05$, Student's *t*-test).

Replacing NADH with NADPH (3 mM) in the enzyme reaction did not significantly change the percentage formation of thiol at 15 min for either PD (0.28 ± 0.03%) or CD (2.90 ± 0.25%).

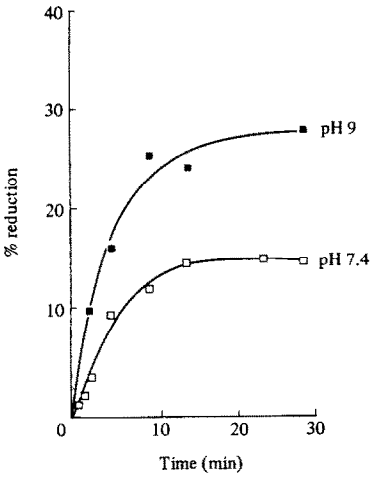

Fig. 3. Time course for the formation of captopril from the *in vitro* reduction of captopril disulfide dimer by (reduced) glutathione at 37° in a 0.1 M potassium phosphate buffer at pH 7.4 (□) and at pH 9.0 (■). Initial concentrations of captopril dimer and glutathione were 1 mM.

DISCUSSION

The reduction of PD in phosphate buffered solution has been shown to be a slow and thermodynamically unfavourable process. Reductants such as tributylphosphine are incapable of reducing PD but can quantitatively reduce CD. This resistance to reduction of PD has been noted before using sodium borohydride [9] and is the main reason why electrolysis has been used to effect reduction of penicillamine disulfides in blood products of patients receiving P therapy [9].

However, it has become clear from the work on captopril that dynamic interconversions of captopril disulfides can take place and complicate the pharmacokinetic profile of this thiol drug. In addition, disulfide metabolites of captopril are present in concentrations many times higher than the parent drug [3]. Since many of these disulfides are not readily excreted by the kidney prior reduction to the parent thiol is necessary for complete removal of the drug [6].

P resistance to reduction may prolong the clearance of the drug and considerably prolong the actions of the drug such as that noted for C. This is consistent with the observation that the offset of action of P in rheumatoid arthritis is at least 3 weeeks [10]. The ability of glutathione, therefore, to reduce PD, albeit weakly, is significant since thiol:disulfide exchange reactions with glutathione are likely pathways for the clearance of PDs.

We have shown here that the reduction of PD by glutathione *in vitro* is some 260 times slower than for captopril disulfide. Mixed disulfide metabolites of P such as penicillamine-cysteine disulfide in which only one sterically hindering B-B-dimethyl group is present are reduced at a faster rate (6-fold) than for PD. Other related metabolites would be expected to behave similarly providing no other factors are involved in this reduction process but the rate is still very slow compared to CDs. However, our studies have shown that thiol reductants are superior to chemical reductants for PD and can be used to at least effect partial thiol exchange of PD.

Tissue clearance of PD is likely however, to be a composite of exchange reactions with glutathione and an enzyme-mediated component similar to that

observed for C [11]. In our studies using a rat red blood cell preparation we found an enzyme-mediated component was indeed contributing to the reduction of PD as it was for CD. At 37° approximately 20–30% of PD was reduced by an enzymic component which contrasted to CD in which approximately 60% was reduced enzymically. The slow rate of reduction of PD was also confirmed *in vivo* in which the rate of CD reduction at 15 min was some 26 times higher than for PD. Although more than one enzyme may be responsible for these reductions the most likely enzyme is thiol-disulfide transhydrogenase [11] which catalyzes the transfer of electrons from glutathione to various disulfides. In this system, oxidized glutathione is regenerated by the NADPH-dependent enzyme glutathione reductase. Although there was no apparent difference in the ability of either NADH or NADPH to affect reduction in our experiments this was not surprising since pyridine nucleotide transhydrogenase is available in tissues to convert NADH to NADPH [11].

Of particular interest, however, was the particularly unfavourable equilibrium constant for this reduction process (as defined by equations 1 and 2) which can be established by allowing the reactants to come to equilibrium. For PD an equilibrium constant of 4.2×10^{-5} was obtained at 37° and pH 7.4 which was not significantly different to the mixed disulfide which contrasts with that for CD which was nearly one thousand fold higher at 3.6×10^{-2}. This constant shows that, at equilibrium, only 0.65% of PD is reduced using equimolar amounts of glutathione and PD. For CD, however, 16% is reduced under identical conditions at equilibrium. This difference in the equilibrium constants for the two thiol exchange reactions is consistent with the differences in the redox potential for the two equivalent electrolytic processes. For PD, however, a small equilibrium constant is also associated with a very slow forward rate constant.

Oxidation of PD and P by oxygen in the incubation medium may be a complicating factor leading to potential side reactions. However, we were not able to distinguish a significant effect on the reduction of PD by glutathione by oxygen although there did appear to be a tendency for a greater degree of reoxidation of P after equilibrium had been established (6 hr incubation time) suggesting a possible oxygen effect. However, this would be unlikely to significantly affect our results on the initial rate of reductions and estimation of the equilibrium constant. Our own experience on CD and C show these species to be quite stable in aqueous solution over a period of several hours in the absence of procedures used to exclude oxygen suggesting that spontaneous oxidation of CD and C is an unlikely complicating factor during the time course of our experiments.

The consequences of this slow rate of reduction and unfavourable equilibrium for PD reinforce the need to develop adequate methods for the measurement of PDs and to assay blood levels of PDs as well as P in patients receiving P therapy.

Acknowledgement—This work was supported by a National Health and Medical Research Foundation Programme Grant.

REFERENCES

1. M. Friedman, *Adv. exp. Med. Biol.* **86B**, 649 (1977).
2. J. C. Grawhall, E. F. Scomen and R. W. E. Watts, *Br. Med. J.* **1**, 585 (1963).
3. O. H. Drummer, P. Worland and B. Jarrott, *Biochem. Pharmac.* **32**, 1563 (1983).
4. O. H. Drummer and B. Jarrott, *Biochem. Pharmac.* **33**, 3567 (1984).
5. B. Fowler and A. J. Robins, *J. Chromatogr.* **72**, 105 (1972).
6. O. H. Drummer, B. S. Workman, P. J. Miach, B. Jarrott and W. J. Louis, *J. Hypertension* **1** (Supp 1), 381 (1983).
7. O. H. Drummer, N. Christophidis, J. D. Horowitz and W. J. Louis, *J. Chromatogr.* **374**, 251 (1986).
8. J. C. Crawhall, E. F. Scowen and R. W. E. Watts, *Br. Med. J.* **1**, 1412 (1964).
9. D. L. Rabenstein and R. Saetre, *Analyt. Chem.* **49**, 1036 (1977).
10. N. Christophidis, D. V. Doyle, M. Ensor and B. Shine, *Ann. Rheumatic Dis.* **42**, 229 (1983).
11. S.-J. Lan, S. H. Weinstein and B. H. Migdalof, *Drug Metab. Dispos.* **10**, 306 (1982).