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NUCLEAR MAGNETIC RESONANCE STUDIES OF THE BINDING OF CAPTOPRIL AND PENICILLAMINE BY SERUM ALBUMIN

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Abstract—The metabolism of the thiol-containing drugs penicillamine (β,β-dimethylcysteine) and captopril (D-3-mercapto-2-methylpropanoyl-L-proline) involves the formation of mixed disulfides, including mixed disulfides with serum albumin. The reactions of penicillamine and captopril with serum albumin in aqueous solution and in intact human blood plasma have been studied by 500 MHz ^{1}H NMR spectroscopy. Penicillamine was found to react rapidly at the albumin-cysteine mixed disulfide bond to form penicillamine-cysteine mixed disulfide and to react more slowly at other albumin disulfide bonds. The amino acid cysteine was found to react with albumin by the same two pathways. In contrast, captopril rapidly associates with albumin to form noncovalent albumin-captopril complexes. Exchange of captopril between its free and noncovalently bound forms takes place on the NMR time scale. On a longer time scale, captopril reacts with albumin by thiol/disulfide interchange reactions. Noncovalently bound captopril displaced lactate from its albumin binding sites, both in aqueous solution and in human plasma. The results demonstrate that ^{1}H NMR is a useful method for characterizing the state of drug molecules in human plasma and for detecting and monitoring perturbations by drugs of delicately balanced binding equilibria involving endogenous small molecules and macromolecules in plasma.

The metabolism of thiol-containing drugs is characterized by the formation of mixed disulfides with amino acids, peptides and proteins, including serum albumin. Mixed disulfides can form spontaneously by thiol/disulfide interchange reactions, which take place via nucleophilic attack by the thiolate anion form of the thiol on disulfide bonds [1, 2]. Serum albumin contains 35 cysteine residues, 34 of which are in intraprotein disulfide bonds [3]. The free cysteine residue, cysteine-34, is found in 25 varieties of albumin from various species, including human serum albumin (HSA)† and bovine serum albumin (BSA) [4]. The sulfur of Cys^{34} is normally present either as the thiol group or in mixed disulfides with cysteine or glutathione (GSH) [5]. Probable sites for reaction of thiol-containing drug molecules with serum albumin include the Cys^{34} mixed disulfide bond (Equations 1 and 2) and albumin disulfide bonds (Equation 3).

$$\mathrm{RSH + AlbSSC \rightleftharpoons AlbSH + RSSC} \quad (1)$$

$$\mathrm{RSH + AlbSSC \rightleftharpoons AlbSSR + CSH} \quad (2)$$

$$\mathrm{RSH + Alb\begin{matrix}S\\|\\S\end{matrix} \rightleftharpoons Alb\begin{matrix}SSR\\\diagdown\\SH\end{matrix}} \quad (3)$$

where RSH represents a thiol-containing drug, CSH represents cysteine, AlbSSC represents the Cys^{34}-cysteine mixed disulfide form of albumin, and $\mathrm{Alb\begin{matrix}S\\|\\S\end{matrix}}$ represents an accessible albumin disulfide bond. Of the two sulfur atoms in the Cys^{34}-cysteine mixed disulfide bond, the cysteine sulfur atom is likely to be more accessible to nucleophilic attack by the thiolate anion form of RSH. Thus, the reaction in Equation 1 is expected to be the more probable pathway for nucleophilic reaction of thiolate groups of drug molecules with the albumin mixed disulfide bond. The reaction in Equation 3 involves the formation of an albumin mixed disulfide, which can in turn react with another molecule of RSH to form the symmetrical disulfide RSSR and another free albumin thiol group.

$$\mathrm{RSH + Alb\begin{matrix}SSR\\\diagdown\\SH\end{matrix} \rightleftharpoons Alb\begin{matrix}SH\\\diagdown\\SH\end{matrix} + RSSR} \quad (4)$$

The objective of the present study was to elucidate the relative importance of these various reaction pathways in the formation of mixed disulfides of penicillamine (PSH) and captopril (CpSH) with serum albumin. Penicillamine (β,β-dimethylcysteine) is used in the treatment of Wilsons's disease, cystinuria, metal intoxication, and rheumatoid arthritis [6]. The metabolism of PSH has been reviewed [6]. Captopril (D-3-mercapto-2-methylpropanoyl-L-proline), an orally active inhibitor of angiotensin I-converting enzyme [7], is effective in reducing arterial blood pressure in most forms of hypertension [8, 9]. Mixed disulfides are formed with

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† Abbreviations: HSA, human serum albumin; BSA, bovine serum albumin; PSH, penicillamine; PSSP, penicillamine disulfide; CpSH, captopril; CpSSCp, captopril disulfide; TBA, *tert*-butyl alcohol; CPMG, Carr–Purcell–Meiboom–Gill; WATR, water attenuation by transverse relaxation; PSSC, penicillamine-cysteine mixed disulfide; PSSG, penicillamine-glutathione mixed disulfide; CSSC, cystine; and CSH, cysteine.

serum albumin in the metabolism of both PSH and CpSH [6, 9, 10].

The reaction of PSH and CpSH with serum albumin has been studied by 500 MHz ^{1}H NMR spectroscopy. Results are presented for PSH and CpSH in intact human plasma, and for the reaction of PSH and CpSH with bovine serum albumin and for the reaction of CpSH with human serum albumin in aqueous solution. Results are also presented for the reaction of cysteine with bovine serum albumin and for the binding of lactate by human serum albumin and bovine serum albumin.

MATERIALS AND METHODS

Chemicals. Penicillamine, cysteine, cystine, lactate, HSA (essentially fatty acid free) and BSA (essentially fatty acid free) were obtained from the Sigma Chemical Co. (St. Louis, MO). Captopril, captopril disulfide (CpSSCp) and *S*-acetylcaptopril were gifts from the Squibb Institute for Medical Research (Princeton, NJ). *S*-Methylcaptopril was prepared from captopril by reaction with silver oxide and methyl iodide [11]. The crude product was purified by silica gel column chromatography (100% ethyl acetate). Evaporation under reduced pressure gave a colorless oil (yield: 81%). The compound was shown to be *S*-methylcaptopril by ^{1}H and ^{13}C NMR and mass spectroscopy.

NMR measurements. ^{1}H NMR spectra were measured at 500 MHz using a Varian VXR-500S spectrometer (Varian Associates, Palo Alto, CA). When the temperature is not specified, spectra were measured at 25°. ^{1}H Chemical shifts were measured relative to either internal *tert*-butyl alcohol (TBA) or alanine but are reported relative to the resonance for the methyl protons of sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The chemical shifts of the ^{1}H resonances for the methyl protons of TBA and alanine are 1.287 and 1.476 ppm vs DSS.

^{1}H NMR spectra were measured by the single pulse method or with the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence [12]. The CPMG pulse sequence $(90°_x - (\tau - 180°_y - \tau)_n$-acquisition) was used to observe selectively resonances for free small molecules in solutions containing HSA or BSA and in intact plasma [13]. Resonances for HSA, BSA and other macromolecules were either reduced in intensity or eliminated completely by transverse relaxation. Typically, a τ of 0.3 msec and a transverse relaxation period ($2\tau n$) of 0.05 to 0.2 sec were used. The intensity of the water resonance in ^{1}H NMR spectra of plasma was reduced with the water attenuation by transverse relaxation (WATR) method [14]. The intensity of the residual water (HOD) resonance in spectra of D_2O solutions was suppressed by presaturation [15].

Preparation of solutions. HSA and BSA solutions were prepared in D_2O or phosphate-buffered D_2O. Typically, the HSA and BSA concentrations were in the 0.5 to 1.0 mM range. The buffer was degassed by bubbling with nitrogen before adding HSA or BSA. Both HSA and BSA contained various small molecule impurities, as indicated by numerous resonances in CPMG ^{1}H NMR spectra of their D_2O solutions. The small molecule impurities were removed by dialysis.

Solutions containing albumin and drug molecules were prepared by transferring 750 μL of an HSA or BSA solution to an NMR tube, and then adding the appropriate volume of a degassed, phosphate-buffered D_2O solution of the drug molecule. Solutions used for time course studies were prepared in the glove box and then sealed with Parafilm to exclude oxygen. A spectrum was collected every 10–15 min for 2–3 hr. Time course data were also measured for a solution containing the drug at the same concentration without the albumin present as a control to ensure that the rate of formation of symmetrical disulfide by air oxidation was negligible.

A solution containing penicillamine disulfide (PSSP), penicillamine-cysteine mixed disulfide (PSSC) and cystine (CSSC) was prepared by reaction of PSH with CSSC to form PSSC and CSH by thiol/disulfide interchange, followed by bubbling with oxygen to convert the CSH and PSH to their disulfide forms [16].

Preparation of plasma. Blood from a healthy volunteer was collected in Becton Dickinson Vacutainers containing Na_2H_2EDTA anticoagulant. Within 10–15 min after collection, the blood was centrifuged at 1050 *g* for 10 min, and then the plasma was drawn off. Degassing the plasma by bubbling with nitrogen to remove dissolved oxygen was not practical due to excessive foaming. To reduce the amount of dissolved oxygen, the plasma was placed in the transfer chamber of an anaerobic glove box immediately after separation from erythrocytes, and then left under nitrogen at 19 mm pressure for 1–2 hr. The plasma was then stored in the glove box (< 100 ppm O_2) until used. This proved sufficient to minimize oxidation of added PSH or CpSH by dissolved oxygen.

RESULTS

PSH and CpSH can react with serum albumin to form mixed disulfides by the reactions in Equations 1–3. Reaction by Equations 1 and 2 would be expected to result in the disappearance of resonances for PSH and CpSH and the appearance of resonances for PSSC or captopril-cysteine (CpSSC) mixed disulfides or cysteine. Reaction by Equation 3 would also result in the disappearance of resonances for PSH and CpSH, but no new resonances would be expected.

Penicillamine. The two nonequivalent methyl groups of PSH give rise to two singlet resonances whose chemical shifts are sensitive to the oxidation state of the thiol group [17]. Spectrum A in Fig. 1 is the methyl region of the CPMG ^{1}H NMR spectrum of a solution containing 1 mM PSH and 1 mM BSA. The spectrum was obtained 29 min after preparation of the solution. The two resonances at 1.562 and 1.464 ppm are from the methyl protons of the reduced form of PSH. The resonance at 1.428 ppm was assigned to one of the two methyl groups of the PS part of PSSC; the resonance for the other methyl group is overlapped by the PSH resonance at 1.562 ppm. Observation of the resonance for PSSC indicated reaction of PSH at the albumin-cysteine

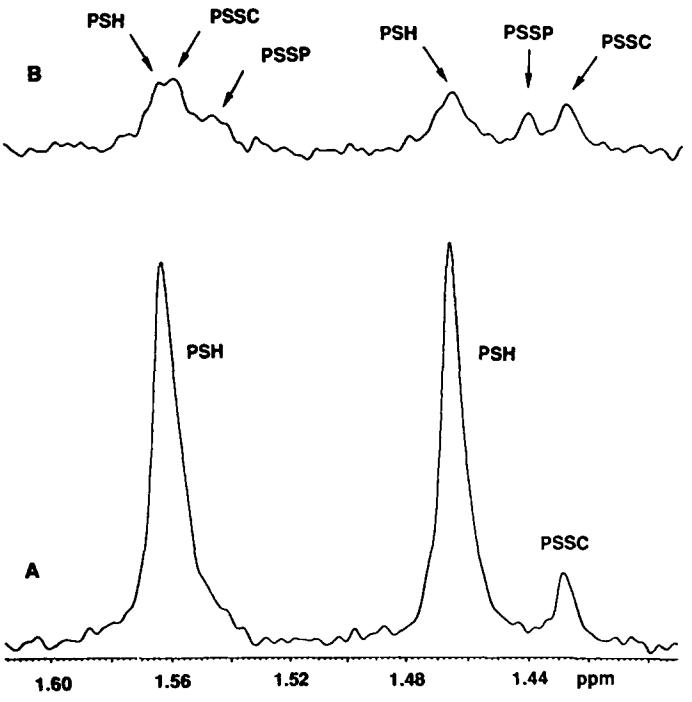


Fig. 1. Portions of 500 MHz CPMG ^1H NMR spectra measured 29 min (A) and 12.5 hr (B) after preparation of a solution that contained 1 mM PSH and 1 mM BSA in D_2O at pD 7.4. The spectra were measured using a transverse relaxation period (2τ) of 0.2 sec in the CPMG pulse sequence.

mixed disulfide bond by Equation 1. However, no resonances were detected for free CSH, indicating no detectable reaction at the Cys^{34} sulfur (Equation 2). Also, no resonances were detected for penicillamine-glutathione mixed disulfide (PSSG) or PSSP.

Spectrum B in Fig. 1 was obtained 12.5 hr after preparation of the solution. The intensity of the PSH resonances had decreased significantly, with no corresponding increase in the intensity of the PSSC resonance. In addition, weak resonances were observed at 1.440 and 1.546 ppm for PSSP. Approximately 5% of the PSH in a D_2O buffered solution that contained no BSA also formed PSSP over this same time period, suggesting that the PSSP in spectrum B was formed by reaction with dissolved oxygen and not by the reaction in Equation 4. The reaction of PSH with AlbSSP to form PSSP was expected to be slow on the basis of reported rate constants for the reaction of PSH with PSSG and PSSC to form PSSP and GSH or CSH [17, 18].

The ratios of the heights of the PSH and PSSC methyl resonances at 1.464 and 1.428 ppm to the height of the TBA resonance (not shown in Fig. 1) are plotted as a function of time in Fig. 2. The ratio for the PSSC resonance remained constant over the 5-hr time period. However, the intensity of the PSH resonance decreased by 80%, presumably by reaction of PSH with disulfide groups in serum albumin (Equation 3).

The 1.38 to 1.60 ppm region of the CPMG ^1H NMR spectrum measured for a sample of human blood plasma 11 min after the addition of 1 mM PSH is shown in Fig. 3. The resonances for albumin and

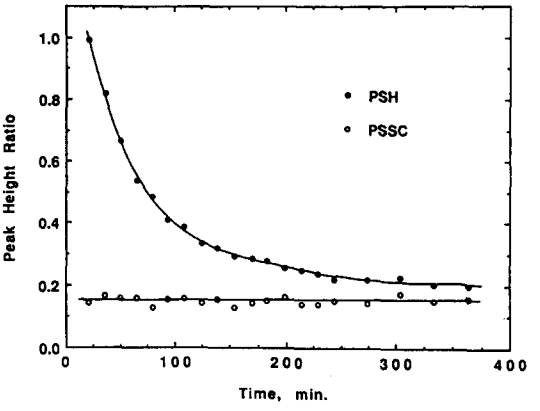


Fig. 2. Ratio of the peak heights of the PSH methyl resonance at 1.462 ppm and the PSSC methyl resonance at 1.426 ppm to the peak height of the TBA methyl resonance at 1.237 ppm vs time after preparation of a solution that contained 1 mM PSH and 1 mM BSA in D_2O at pD 7.4.

other macromolecules in plasma were selectively eliminated from the spectrum by transverse relaxation [13]. The resonances at 1.477 and 1.462 ppm for the methyl protons of alanine are the only resonances in the 1.38 to 1.60 ppm region from endogenous small molecules. The other resonances were assigned to the methyl protons of PSH, PSSC and PSSP by spiking a plasma sample with a solution containing

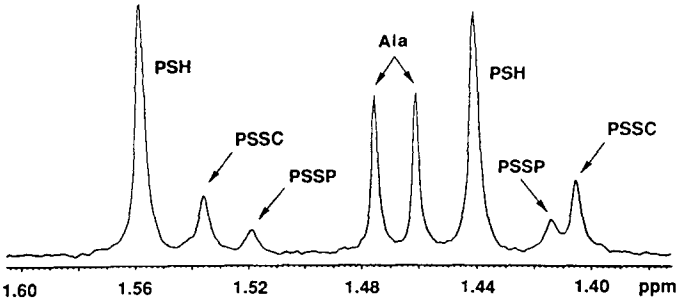


Fig. 3. A portion of the 500 MHz CPMG ^{1}H NMR spectrum measured for a sample of human blood plasma 11 min after the addition of 1 mM PSH. The pH of the sample was 7.4. A transverse relaxation period of 0.20 sec was used in the CPMG pulse sequence and the water resonance was suppressed by the WATR method.

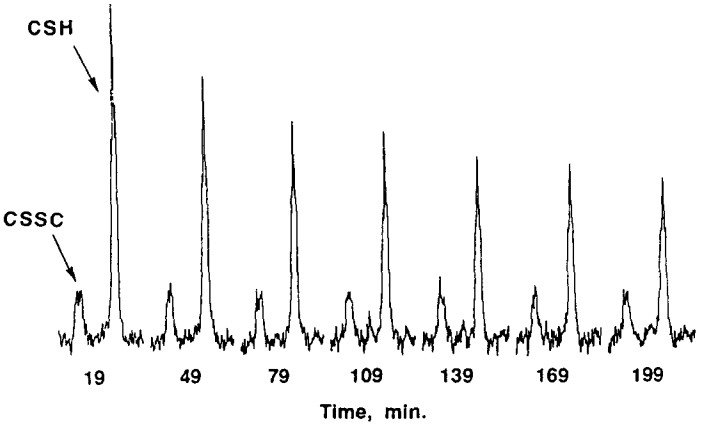
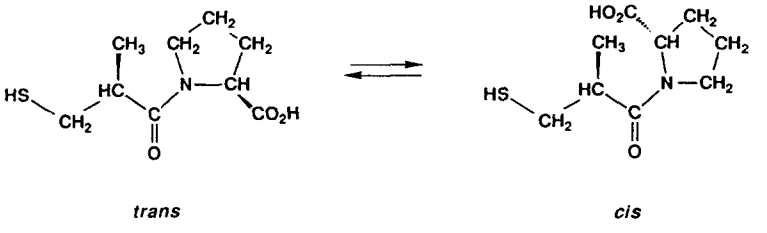


Fig. 4. The 3.85 to 4.20 ppm region of 500 MHz CPMG ^{1}H NMR spectra measured as a function of time after preparation of a solution that contained 2 mM cysteine and 1 mM BSA in D_2O at pD 7.4.

PSSP, PSSC and CSSC. The differences in the chemical shifts of several resonances in Figs. 1 and 3 are due to small differences in the protonation states of the ammonium groups of PSH, PSSC and PSSR in the two samples; the pK_a values of the ammonium groups are higher in D_2O solution. The intensity of the PSH resonance decreased with time, but less rapidly than shown in Fig. 2 for the PSH/BSA solution, e.g. the PSH:TBA ratio decreased by 30% over a 160-min time period compared to 70% over the same time period for the PSH/BSA sample.

Cysteine. Similar results were obtained from studies of the reaction of cysteine with BSA. The 3.8 to 4.2 ppm region of a series of 500 MHz CPMG ^{1}H NMR spectra measured as a function of time for a solution containing 2 mM cysteine and 1 mM BSA is shown in Fig. 4. The resonances are for the $C_\alpha H$ protons of CSSC (4.11 ppm) and CSH (3.97 ppm). The resonance for CSSC was observed within 5 min



Scheme 1.

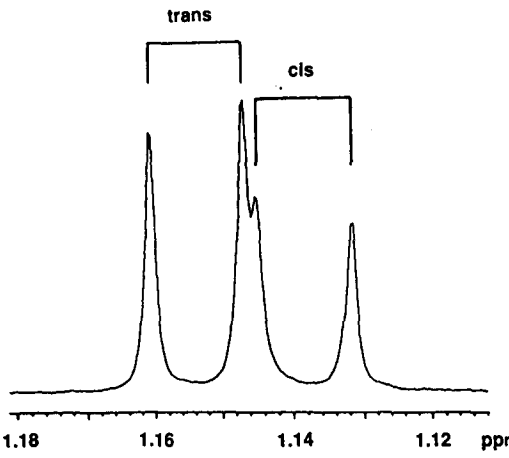


Fig. 5. Methyl resonances in the 500 MHz ^{1}H NMR spectrum of CpSH in D_2O at pD 7.35.

of the addition of CSH to BSA, which suggests that CSH reacts rapidly with the BSA-cysteine mixed disulfide (Equation 1). The intensity of the resonance for CSSC remained essentially constant, whereas that for the CSH resonance decreased by ~50% over a period of 4 hr, presumably by reaction with other disulfide bonds of BSA (Equation 3).

Captopril. In aqueous solution, CpSH exists as an equilibrium mixture of the *trans* and *cis* isomers (Scheme 1). The ^{1}H NMR spectrum of CpSH consists of resonances from two highly coupled spin systems: the $CH_2CH_2CH_2CH$ spin system of the proline residue and the CH_2CHCH_3 spin system of the 3-mercapto-2-methyl-1-oxopropyl group [19]. Interconversion between the *trans* and *cis* isomers by rotation around the C—N bond is slow on the NMR time scale (the lifetimes of the *trans* and *cis* isomers are ~270 and 170 sec, respectively, at 25°) [19, 20], and thus there are two separate sets of resonances for each spin system, one for the *trans* isomer and one for the *cis* isomer. To illustrate, the two sets of resonances for the methyl protons of CpSH are shown in Fig. 5. The relative intensities of the two doublets were 0.61:0.39, from which a value of 1.6 was obtained for the *cis* $\rightleftharpoons$ *trans* equilibrium constant. The methyl resonances of CpSH were used to study its interaction with serum albumin; they were the most intense resonances for CpSH and there were no interfering resonances in the 1.1 to 1.2 ppm region of the CPMG ^{1}H NMR spectrum of plasma.

The 0.74 to 1.52 ppm region of the CPMG ^{1}H NMR spectrum of human plasma is shown in Fig. 6A. Resonances were observed for protons of several endogenous small molecules, including the methyl protons of alanine (1.477 and 1.462 ppm), lactate (1.325 and 1.311 ppm) and valine (1.038, 1.024, 0.984 and 0.970 ppm) [13]. The broad resonance at 1.267 ppm has been assigned to the methylene protons of chylomicrons and very low density lipoprotein; those at 0.873 and 0.837 ppm to the methyl protons of high density and low density lipoprotein [21]. The resonance at 1.237 ppm is for TBA, which was added as a chemical shift reference.

Spectrum B in Fig. 6 was obtained after addition of 10 mM CpSH to the plasma. Resonances were observed in the 1.1 to 1.2 ppm region for the methyl protons of CpSH; however, their intensity was much less than expected if the CpSH were all free in solution. Also, the intensity of the resonance for the *trans* isomer was less than that for the *cis* isomer, opposite to the relative intensities in free solution (Fig. 5). The weak resonances in the region from 1.18 to 1.20 ppm in spectrum B are for the methyl protons of CpSSCp [22], which is apparently formed by reaction of CpSH with dissolved oxygen.

To determine if the low intensity of the resonances observed for CpSH in plasma was a result of binding by serum albumin, ^{1}H NMR spectra were measured for solutions containing CpSH and HSA. Spectrum A in Fig. 7 is the CPMG ^{1}H NMR spectrum obtained for a solution containing 4 mM CpSH and 1 mM alanine; spectrum B is for a solution containing 4 mM CpSH, 1 mM alanine and 1 mM HSA. All resonances for CpSH are absent from spectrum B, indicating that CpSH is associated with HSA. It was determined by ulrafiltration using a membrane with a molecular weight cutoff of 3000 that 41% of the CpSH was free in solution. However, as described below, resonances were not observed for the free CpSH due to exchange of CpSH between its free and bound forms. At higher concentrations, broadened resonances were observed for CpSH. For example, portions of CPMG ^{1}H NMR spectra measured as a function of temperature for a solution containing 9.90 mM CpSH, 1 mM alanine, 0.45 mM HSA and 0.05 mM TBA are shown in Fig. 8. The CpSH and HSA concentrations were similar to those in the plasma sample used to obtain spectrum B in Fig. 6. The intensity of the CpSH resonances was considerably lower than expected for the relative concentrations of CpSH and alanine, and the relative intensities of the *trans* and *cis* resonances at 25° were similar to those observed for CpSH in plasma.

The methyl resonances for CpSH in both plasma and in solution with HSA were broad, with the *trans* resonance broadened more than the *cis* resonance. The sharpness of the alanine resonances indicates that the broadening of the CpSH resonances was not due to solution viscosity or other nonspecific effects. Rather, the dependence of the line broadening on temperature (Fig. 8) indicates that the broadening of the CpSH resonances was due to the exchange of CpSH between free and HSA-bound forms. The correlation time of bound CpSH will be similar to that of HSA and thus the resonances of bound CpSH will be broad while the resonances for free CpSH will be sharp. If exchange of CpSH between its free and bound forms were slow on the NMR time scale (lifetime $> \sim 2$ sec in each form), sharp resonances would be observed for the free CpSH while those for bound CpSH would be eliminated from the CPMG ^{1}H NMR spectrum by transverse relaxation. However, the absence of resonances for CpSH in spectrum B in Fig. 7 indicates this is not the case. At faster rates of exchange, an exchange-broadened resonance would be observed for free CpSH while the resonance for

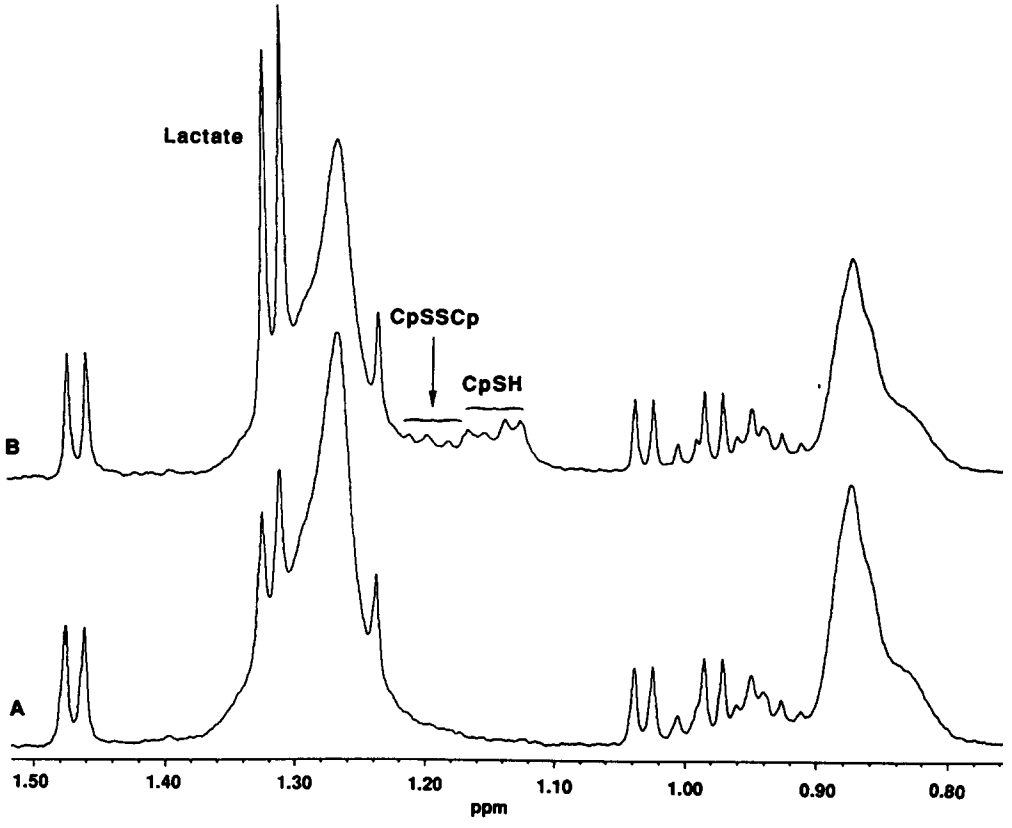


Fig. 6. Portions of 500 MHz CPMG ^{1}H NMR spectra of (A) human blood plasma and (B) human plasma containing 10 mM CpSH. Additional resonance assignments are given in the text.

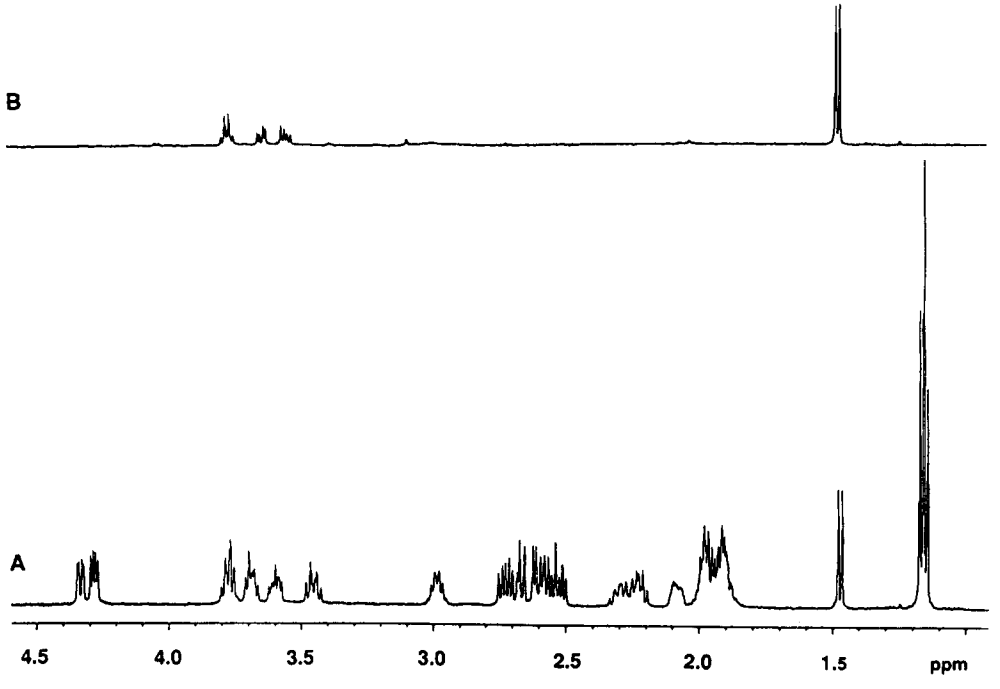


Fig. 7. 500 MHz CPMG ^{1}H NMR spectra of (A) 4 mM CpSH and 1 mM alanine and (B) 4 mM CpSH, 1 mM alanine and 1 mM HSA.

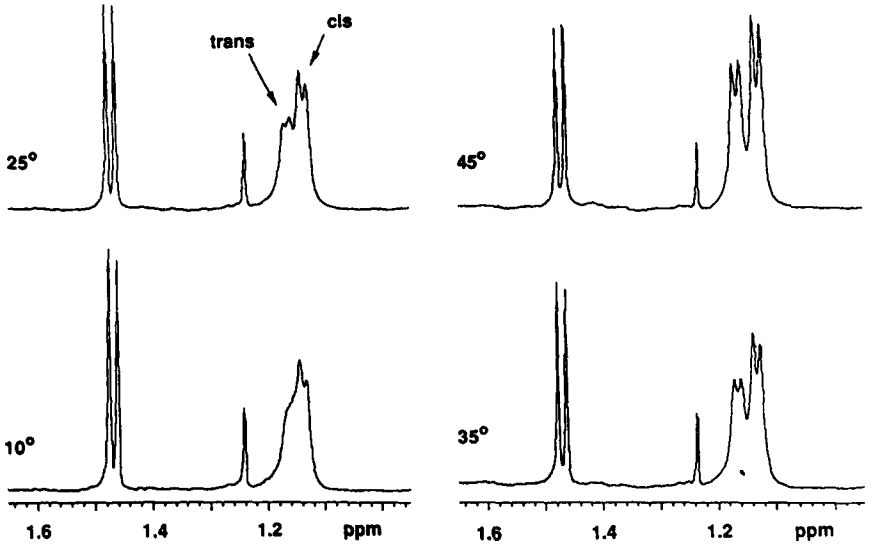


Fig. 8. Portions of 500 MHz CPMG ^{1}H NMR spectra measured as a function of temperature for a solution that contained 9.90 mM CpSH, 0.45 mM HSA, 1 mM alanine and 0.05 mM TPA. A transverse relaxation period of 0.05 sec was used in the CPMG pulse sequence.

bound CpSH would be eliminated by transverse relaxation. At even faster rates of exchange, an exchange-averaged resonance would be observed for the free and bound CpSH. In the case of an exchange-broadened resonance for free CpSH, the line broadening would increase as the temperature is increased, whereas in the case of an exchange-averaged resonance, the line broadening would decrease with an increase in temperature. The CpSH resonances in Fig. 8 sharpened as the temperature was increased, indicating that the resonances were an exchange average of resonances for free and bound CpSH. However, because the exchange averaged resonance for the *trans* isomer was broadened more than that for the *cis* isomer, the exchange averaged *trans* resonance was of lower intensity due to more attenuation by transverse relaxation.

The binding of CpSH by BSA was also studied. As observed for the CpSH/HSA system, resonances for the *trans* isomer were completely absent from CMPG ^{1}H NMR spectra of solutions containing 4 mM CpSH and 1 mM BSA. However, broadened resonances were observed for the *cis* isomer. This was also the case at higher concentrations of CpSH (up to 30 mM). The intensity of the *cis* resonance for CpSH decreased slowly with time, e.g. the intensity of the *cis* resonance in the spectrum of the solution containing 4 mM CpSH and 1 mM BSA decreased by 10% over a period of 140 min. Also, there were no detectable resonances in the 3.9 to 4.2 ppm region for cysteine or the cysteine part of captopril-cysteine mixed disulfide, and there were no resonances in the 1.15 to 1.20 ppm region for CpSSCp.

The intensity of the lactate resonance in the spectrum obtained after addition of CpSH to plasma (spectrum B in Fig. 6) was increased relative to that of the alanine resonance. To determine if this was also a result of the binding of CpSH by serum albumin, CPMG ^{1}H NMR spectra were measured for solutions containing 1 mM HSA, 8 mM lactate and 0, 2, 4, and 8 mM CpSH. Alanine (1 mM) was added as an intensity reference. Portions of the spectra are plotted in Fig. 9. The ratio of the intensities of the lactate and alanine resonances in spectrum A was much less than the ratio of their concentrations, indicating that a large fraction of the lactate was bound by HSA. In spectra B–D, the intensity of the lactate resonance increased as the concentration of CpSH was increased, indicating that lactate was displaced from its HSA binding sites by CpSH. Similar results (not shown) were also obtained in parallel studies of the binding of lactate and CpSH by BSA.

The above results indicate that CpSH is bound by HSA, that exchange of CpSH between its free and bound states takes place on the NMR time scale (lifetimes of the two states $< \sim 0.01$ sec), and that CpSH displaces lactate from its HSA binding sites. Two possible mechanisms for the interaction of CpSH with HSA are covalent bond formation by thiol/disulfide interchange reactions and noncovalent binding of CpSH in one of the several ligand binding sites on HSA [3, 23, 24]. To determine if the interaction involves the thiol group of CpSH, similar HSA binding and lactate displacement experiments were performed with the thiol-blocked derivatives *S*-methylcaptopril ($CpSCH_3$), *S*-acetylcaptopril ($CpSCOCH_3$) and CpSSCp. In each case, results similar to those described above for the CpSH/HSA system were obtained. For example, resonances for $CpSCH_3$ in CPMG ^{1}H NMR spectra of solutions containing $CpSCH_3$, HSA and alanine were less intense than expected, and the resonances for the *trans* isomer of $CpSCH_3$ were less intense than those

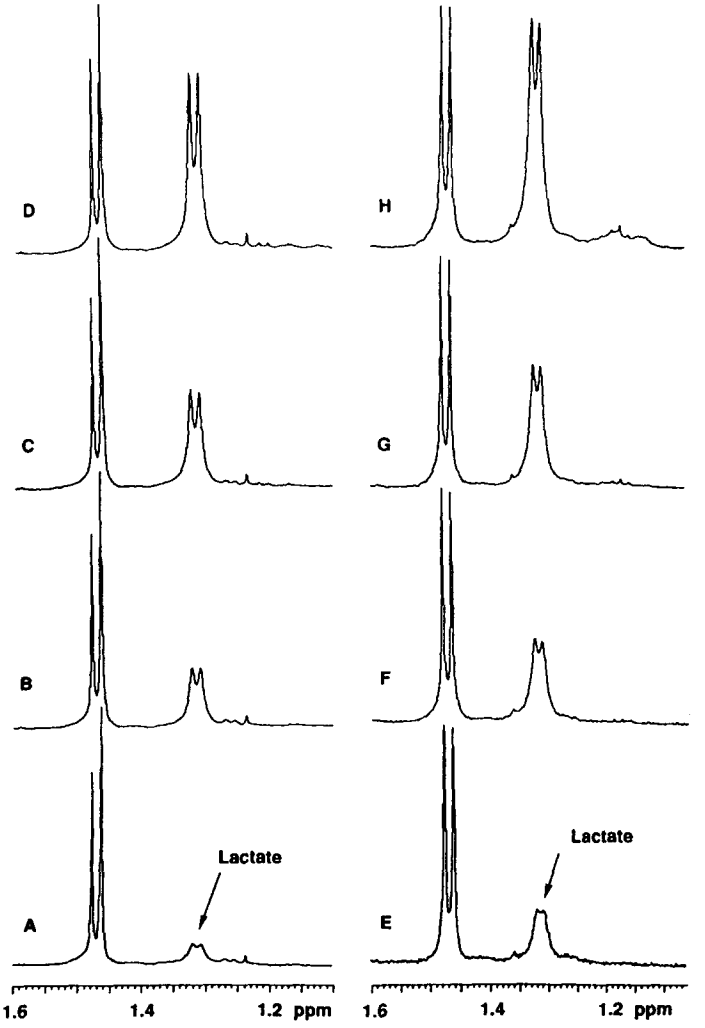


Fig. 9. Portions of CPMG ^{1}H NMR spectra of solutions that contained 1.0 mM alanine, 8.0 mM lactate. 1 mM HSA and either CpSH (A–D) or *S*-methylcaptopril (E–H). The CpSH concentrations were 0, 2, 4 and 8 mM for A–D, respectively. The *S*-methylcaptopril concentrations were 0, 2, 4 and 8 mM for E–H, respectively. The doublet resonances of 1.462 and 1.477 ppm are from the methyl protons of alanine.

for the *cis* isomer. Also, the intensity of the lactate resonance increased when $CpSCH_3$ was added to solutions containing lactate, HSA and alanine (spectra E–H in Fig. 9).

Lactate. The increase in the intensity of the lactate resonance when CpSH was added to human plasma (Fig. 6) is evidence that a fraction of plasma lactate is not visible in the CPMG ^{1}H NMR spectrum of human plasma. The intensity of the lactate resonance is also reported to increase in spin-echo ^{1}H NMR spectra of plasma after the addition of ammonium chloride [21]. The results in Fig. 9 provide evidence that the NMR-invisible lactate is bound by HSA. To obtain additional information about the interaction of lactate with HSA, saturation transfer NMR experiments were performed. The experiments involved selective irradiation with the decoupler set at a specific chemical shift for 5 sec, followed by measurement of the ^{1}H NMR spectrum. Results from two experiments are plotted in Fig. 10. The top data set is for a solution of lactate and alanine. The ratio of the intensities of the lactate and alanine methyl resonances is plotted vs the chemical shift setting of the decoupler. Although there is some scatter, the intensity ratio is essentially independent of decoupler frequency. The bottom data set is for a solution that contained lactate, alanine and HSA. In this experiment, HSA resonances at the chemical shift of the decoupler pulse were selectively saturated. This saturation was then transferred by cross relaxation to other protons, including the methyl protons of bound lactate, if they were linked by dipole–dipole interactions to the saturated protons [25]. Exchange of lactate between its free and bound

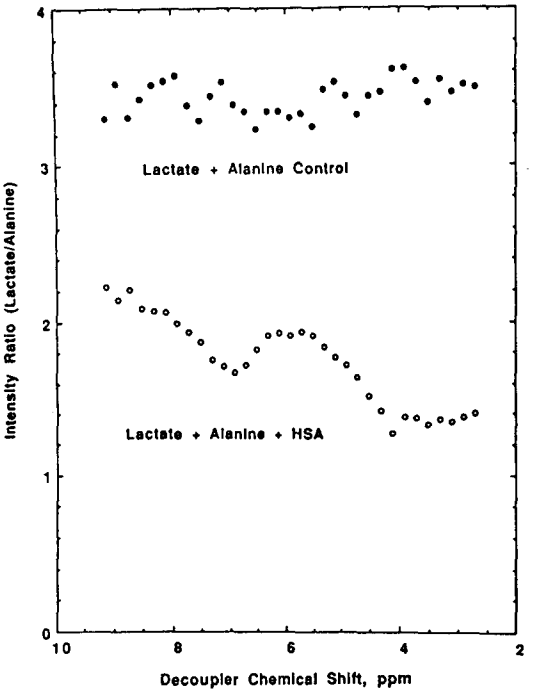


Fig. 10. Ratio of the peak heights of the lactate and alanine methyl resonances as a function of the chemical shift of the decoupler in saturation transfer experiments. The decoupler pulse was applied for 5 sec and then the spectrum was measured. The top data set is for a solution that contained 3.7 mM lactate and 1 mM alanine in D_2O; the bottom data set is for a solution that contained 3.7 mM lactate, 1 mM alanine and 0.5 mM HSA in D_2O.

forms will result in the transfer of saturation to free lactate, with the result that the intensity of the resonance for free lactate will decrease. A ratio of 2.71 was obtained for the intensities of the lactate and alanine resonances with the decoupler set at a chemical shift of 40 ppm, where there are no resonances for HSA. This intensity ratio was less than observed for the lactate/alanine solution due to the binding of some of the lactate by HSA. However, the intensity ratio was decreased even further when the decoupler was set at chemical shifts in the 2.5 to 9 ppm range (bottom data set, Fig. 10), and the intensity ratio was a function of the decoupler setting in this chemical shift range. These results indicate transfer of saturation by exchange of lactate between its free and bound forms.

DISCUSSION

Most drugs are associated to some extent with macromolecules, including serum albumin, as they are carried by the circulating blood from their sites of absorption to their sites of action [26]. Albumin binding influences the fate of drugs in the body, since only the unbound or free drug diffuses through capillary walls, reaches the site of drug action, and is subject to elimination from the body. The above results indicate that both PSH and CpSH interact with serum albumin, but that the nature of the interaction is quite different.

Penicillamine. The observation of resonances for PSSC in Figs. 1 and 3 indicates that PSH reacts rapidly with the albumin-cysteine mixed disulfide bond in both aqueous solution and in plasma (Equation 1). The time course data in Fig. 2 for the disappearance of the PSH resonances suggests that PSH also reacts with other disulfide bonds of albumin (Equation 3), but that this reaction is much slower than reaction with the albumin-cysteine mixed disulfide bond. The results also indicate there is little if any PSSP formed by reaction of PSH with albumin-penicillamine mixed disulfide formed by the reaction in Equation 3. This is as expected from previous studies which show that the kinetics of the reaction of PSH with penicillamine-cysteine and penicillamine-glutathione mixed disulfides to form PSSP are extremely slow due to steric effects involving the methyl groups of PSH [17, 18]. Although the PSH concentrations used in this study were higher than the concentration in the blood of patients receiving PSH, these qualitative conclusions are nevertheless applicable to reactions of PSH with HSA *in vivo*. The kinetics of thiol/disulfide interchange reactions of PSH are second-order: first order in PSH and in the disulfide [17, 18]. Thus, the half-time for reaction of PSH with HSA by thiol/disulfide interchange is inversely proportional to the HSA concentration, but independent of PSH concentration.

The PSH and PSSC resonances in Fig. 1A are sharp and they remained sharp as the intensity of the PSH resonances decreased with time, consistent with slow interchange of PSH between its free and albumin bound forms on the NMR time scale. This is as expected for covalent binding via disulfide bond formation [17, 18].

The results in Fig. 4 indicate that cysteine also reacts relatively rapidly with the albumin-cysteine mixed disulfide bond, followed by a slower reaction with other albumin disulfide bonds.

Captopril. The binding of CpSH by serum albumin was somewhat more complicated than the binding of PSH. The absence of resonances for CpSH in ^1H NMR spectra measured immediately after preparation of solutions containing 1 mM HSA and 4 mM CpSH indicates that CpSH associates rapidly with HSA. Observation of the same results for *S*-methylcaptopril and *S*-acetylcaptopril is evidence that the association involves noncovalent interactions between CpSH and HSA, rather than thiol/disulfide interchange reactions as described by Equations 1–3. However, the slow decrease in the intensity of the resonances for the *cis* isomer of CpSH with time indicates that CpSH also reacts with albumin by a much slower process, presumably involving formation of covalent bonds by thiol/disulfide interchange reactions. Albumin-captopril mixed disulfides have been reported to be metabolites of captopril [9, 10].

The temperature dependence of the resonance line broadening in Fig. 8 for the CpSH methyl protons indicates that both the *trans* and *cis* isomers are bound by HSA, and that interchange between their free and noncovalently bound forms takes place

on the NMR time scale. Thus, resonances for CpSH are broadened by exchange, the result of which is that they are reduced in intensity or absent from ^1H NMR spectra measured for solutions of CpSH and HSA by the CPMG pulse sequence. The differential broadening of the *trans* and *cis* resonances indicates that the rate of interchange between the free and noncovalently bound forms is faster for the *cis* isomer.

The nature of the noncovalent binding of CpSH by HSA cannot be established from the results reported here; however, displacement of lactate by CpSH suggests that CpSH competes with lactate for specific binding sites on HSA or that binding of CpSH causes changes in the conformation of HSA which lowers its affinity for lactate. It is of interest to note that binding of penicillamine by HSA had little effect on the intensity of the lactate resonance in CPMG ^1H NMR spectra of human plasma.

Lactate. Although the binding of a variety of endogenous molecules by HSA has been characterized, the binding of lactate apparently has not. In fact, we were unable to find any reports of the binding of lactate by HSA. However, the results in Figs. 6 and 8 indicate that a large fraction of the lactate in plasma is bound by HSA. Furthermore, the results suggest that exchange of lactate between the free and bound forms is slow on the NMR time scale.

The principal regions for ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA [24]. For example, recent crystallographic results show that 2,3,5-triiodobenzoic acid (TIB) binds to both subdomains by interaction of the hydrophobic part of the aromatic ring of TIB with hydrophobic side chains of several amino acid residues, including phenylalanine [24]. The carboxylate group of TIB interacts with positive centers on the side chains of arginine and/or lysine amino acid residues.

The results of the saturation transfer experiments (Fig. 10) are consistent with the binding of lactate in such an environment. Transfer of saturation to the methyl protons of lactate is greatest when the HSA spectrum is irradiated in the region of 7 ppm and over the 2.5 to 4 ppm region. Resonances for protons of aromatic side chains are found in the 7 ppm region [27], which suggests that hydrophobic interactions between the aromatic side chains of phenylalanine residues and the methyl group of lactate are involved in the binding of lactate. Resonances for $C_\alpha H$ protons and side chain protons of other amino acids are found in the 1 to 4.5 ppm region [27], which suggests that noncovalent binding of lactate also involves interactions with the hydrophobic side chains of other amino acids, as found in the binding of TIB and several other small molecules [24].

The dip at 4.1 ppm in the intensity ratio vs decoupler setting plot is due to the transferred nuclear Overhauser effect (NOE) [28] and provides additional evidence for exchange of lactate between its free and bound forms. Saturation of the resonance for the CH proton of bound lactate at 4.1 ppm results in a negative NOE between the CH and CH_3 protons, which is then transferred to the resonance for free lactate by dissociation of the bound lactate.

Summary. The results of this study have provided detailed information about the various reactions involved in the interaction of the thiol-containing drugs penicillamine and captopril and the amino acid cysteine with serum albumin, both in aqueous solution and in intact human plasma. The results also demonstrate that lactate is bound by serum albumin, and they provide information about interactions involved in the binding.

The results also demonstrate that, with the chemical shift dispersion of high field NMR spectrometers, ^1H NMR spectroscopy is a useful method for characterizing the state of drug molecules in human plasma. Human plasma is a complex fluid, with many delicately balanced equilibria involving the binding of small molecules by macromolecules. The results presented here demonstrate that perturbation of these equilibria, e.g. by competitive binding of drug molecules, can be detected by ^1H NMR. Since resonances are observed simultaneously for many of the components of plasma, multiple equilibria can be monitored simultaneously and thus unsuspected interactions can be detected, e.g. the competitive binding of CpSH and lactate by HSA which was detected in the present study.

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