THE *IN VITRO* LOSS OF PENICILLAMINE IN PLASMA, ALBUMIN SOLUTIONS,
AND WHOLE BLOOD: IMPLICATIONS FOR PHARMACOKINETIC STUDIES OF PENICILLAMINE

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Summary

The recent development of a high performance liquid chromatography
assay method for the analysis of penicillamine in biological
samples such as plasma, whole blood, and urine has provided a
specific and sensitive assay method to aid in the study of
penicillamine pharmacokinetics. Several investigators have reported
measuring the plasma concentration of penicillamine. Some of these
investigators have indicated that the plasma must be assayed
immediately. However, such restrictions can limit the feasibility
of a pharmacokinetic study. The results of this paper demonstrate
the instability of penicillamine in plasma, albumin solutions, and
whole blood. The rate of loss of penicillamine was shown to be
influenced by the concentration of albumin. As a result of the
significant loss of penicillamine over a short period of time,
plasma or whole blood samples must be deproteinated immediately upon
collection to avoid the loss of reduced penicillamine. Methods are
presented for the preparation of biological samples so that the
oxidation of penicillamine is prevented and the samples can be held
for several days prior to analysis.

Penicillamine has been used therapeutically since 1954 when it was first
introduced by Walshe (1) for the treatment of Wilson's disease. Since that time
a number of other uses for penicillamine have been advocated including the
treatment of cystinuria (2), rheumatoid arthritis (3), metal intoxication (4),
primary billiary cirrhosis (5), chronic hepatitis (6), and hyperbilirubinemia
(7). Lyle recently has published an excellent review of penicillamine (8).

A detailed human pharmacokinetic study of penicillamine has not been

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reported primarily due to the lack of a specific and sensitive assay for penicillamine in biological fluids. Some investigators have reported pharmacokinetic studies of penicillamine using radiolabeled drug (9-14). However these studies are inappropriate as the radioactivity represents both metabolized and unchanged drug. Analytical methods for the analysis of penicillamine such as colorimetric (15,16), gas chromatographic (17), immuno-logical (18,19) and amino acid analyzer (20) have been developed. These methods have limitations such as non-specificity, low sensitivity, or require complex manipulations of the samples which renders their application in the analysis of pharmacokinetic samples inappropriate. The recent development of a high per-formance liquid chromatography (HPLC) method by Saetre and Rabenstein (21) for the analysis of penicillamine using an electrochemical detector has provided a simple, specific and sensitive analytical tool suitable for pharmacokinetic samples. We have used this HPLC method in our laboratory with some modifi-cations to assess the stability of the reduced form of penicillamine in plasma, albumin solutions, and whole blood. The results of these studies and the corresponding implications for proposed pharmacokinetic studies are presented.

Methods

The stability of penicillamine in plasma, whole blood, and buffered albumin solutions was studied in vitro using plasma obtained from the American National Red Cross Blood Service (single donor source, citrate-phosphate anticoagulant used), whole blood obtained from a normal volunteer (ethylene diamine tetracetic acid disodium (EDTA) anticoagulant used) and normal human serum albumin 25% obtained from the American National Red Cross Blood Service.

The various concentrations of albumin solutions studied were prepared by diluting the normal human serum albumin 25% to the appropriate concentration using an isotonic pH=7.4 imidazole buffer (imidazole 4.3 g., HCl 0.1 M. 225 ml., EDTA 250 mg., deionized water q.s. to 250 ml.).

All of the experiments were conducted at controlled temperatures. An alli-quot of a biological fluid (25 ml.) was pipetted into a water jacketed flask. The flask was maintained at a constant temperature (10, 25, 37, or 55°C.) by circulating controlled temperature water through the water jacket from a Brink-mann MGW Lauda refrigerated water bath (model RC-3) equipped with a MGW Lauda (model T-2) temperature controller (Brinkmann Instruments, Inc. Westbury, N.Y. 11590). The temperature of the biological medium was allowed sufficient time to equilibrate to the controlled temperature before the addition of peni-cillamine. The biological medium was constantly stirred using a teflon coated magnetic stirring bar.

The experiments were started by the addition of a concentrated solution of D-penicillamine (Aldrich Chemical Co., Milwaukee, Wisconsin 53233) to the flask. At timed intervals following the addition, samples were withdrawn from the flask. For the initial rate studies, samples (1.0 ml.) were obtained at .25, 1, 2, 3, 4, 5, 7, 9, and 11 minutes after the penicillamine addition. For the other studies, samples (2.0 ml.) were obtained at 1, 10, 20, 30, 40, 50, 60, 70, 80, and 90 minutes after addition of the concentrated penicillamine solution.

The samples were treated immediately to cause a precipitation of the proteins and a decrease in pH. The plasma samples were added to test tubes containing a trichloroacetic acid solution (15% w/v) (0.25 ml. per ml. of sample). The albumin samples were added to test tubes containing a metaphos-phoric acid solution (500 g./L.) (0.25 ml. per ml. of sample). The whole blood samples were added to test tubes containing an EDTA solution (200 mg./L.) (an equal volume to that of the sample) and the blood hemolyzed. Immediately an alliquot of a metaphosphoric acid solution (500 g./L.) (0.5 ml. per ml. of

original sample) was added to the hemolyzed blood sample to precipitate the proteins. All of the precipitated samples were agitated to ensure complete precipitation and mixing. The initial rate samples were centrifuged at 15000 rpm (Eppendorf Micro Centrifuge model 5412, Brinkmann Instruments, Inc. West-bury, N.Y. 11590) for one minute. The supernatant fluids of these samples were decanted into clean test tubes and injected directly onto the HPLC. All of the other samples were centrifuged at 2500 rpm (Sorvall model RC-3 refrigerated centrifuge, Dupont Instruments, Newtown, Ct. 06470) for 6 minutes. The resul-ting protein free supernatant fluids were decanted and then filtered through a 0.45 μ. membrane filter (13 mm. Swinnex with HATF01300 membrane filter, Milli-pore Corporation, Bedford, Mass, 01730) into clean test tubes. The concen-tration of penicillamine remained unchanged in the protein free supernatant of samples that were treated by the above methods. For penicillamine analysis 20 μl. alliquots of the samples were injected onto the HPLC system.

For the plasma and 5% albumin solutions studied at the three different temperatures (10, 25, and 37°C.) the theoretical initial concentration after the addition of the concentrated penicillamine solution was 19.6 μg./ml. The theo-retical initial concentration was 38.3 μg./ml. for the whole blood study and was 19.5 μg./ml. in the 1, 3, 5, 7% albumin study. Initial rate studies were completed using initial concentrations of approximately 6, 23, and 200 μg./ml. penicillamine for the 5% buffered albumin solutions only.

The HPLC method and electrochemical detector utilized for these studies are a modification of the HPLC method and detector reported previously by Saetre and Rabenstein (21). Two stainless steel columns packed with Zipax SCX (Dupont Instruments, Wilmington, Del 19898) (0.41 x 5 cm. and 0.41 x 30 cm.) were used as the guard and analytical columns. A citrate/phosphate buffer (citric acid 0.03 M., and sodium dibasic phosphate 0.01 M.) was used as the mobile phase. The flow rate was maintained at 2.6 ml./min. and resulted in a back pressure of 300-400 psi. A mercury gold amalgam working electrode, Ag/AgCl reference elec-trode, and a glassy carbon auxiliary electrode were used in the electrochemical detector's transducer (model TL-6A Bioanalytical Systems, Inc. West Lafayette, Indiana 47906). The LC-4 amperometric controller (Bioanalytical Systems, Inc., West Lafayette, Indiana 47906) was used to maintain the potential of the working electrode at +0.01 V. vs. the Ag/AgCl reference electrode. Full details of the HPLC system will be published elsewhere.

The electrolysis method for the determination of total penicillamine content utilized the electrolysis cell described by Saetre and Rabenstein (21). A Wenking potentiostat (model 61 RS Brinkmann Instruments, Inc. Westbury, N.Y., 11590) with an external 270 ohm resister between the working and reference electrodes was used as a galvanostat to supply 6.4 mA of current to the electro-lysis cell for the reduction of penicillamine disulfides to penicillamine. A citric acid solution (0.4 M.) was used to dilute the samples for the electro-lysis procedure (equal volume used to that of the sample electrolyzed).

<u>Results</u>

The <u>in vitro</u> concentrations of penicillamine in plasma or albumin solutions have been shown to rapidly decrease with time. At the concentrations used, the rate of this loss was shown to be temperature dependent. Figure 1 shows the loss of penicillamine at three temperatures that occurred when penicillamine was added to normal human plasma <u>in vitro</u>. Figure 2 shows the loss of penicillamine in a 5% albumin solution at the same three temperatures. (All figures were computer drawn. The axis numerals do not represent the data accuracy).

The observed loss of penicillamine in these experiments is not due to the experimental conditions. The results of simultaneous control experiments using
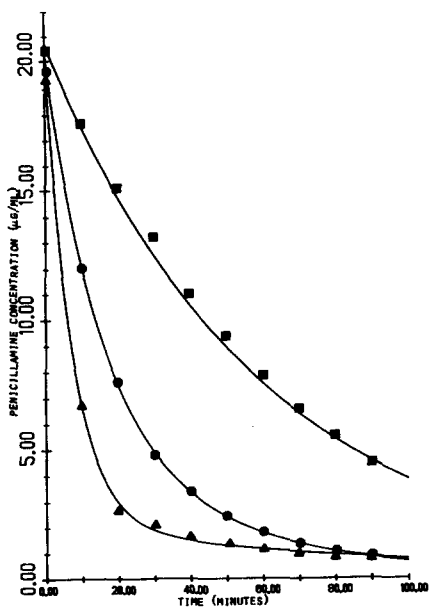
FIG. 1
The Loss of Penicillamine in Normal
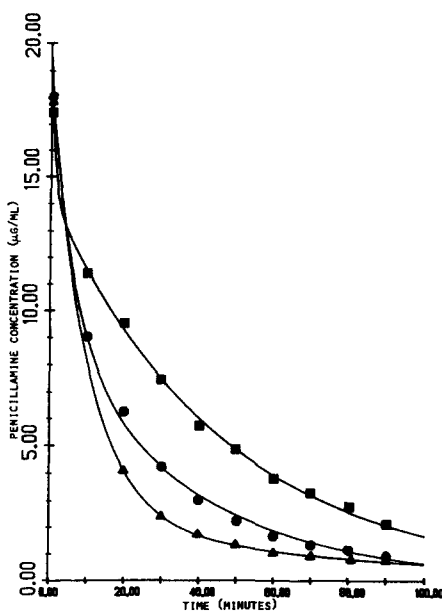Human Plasma. squares=10°C.,
circles=25°C., triangles=37°C.

FIG. 2
The Loss of Penicillamine in a
5% Albumin Solution. squares=10°C.,
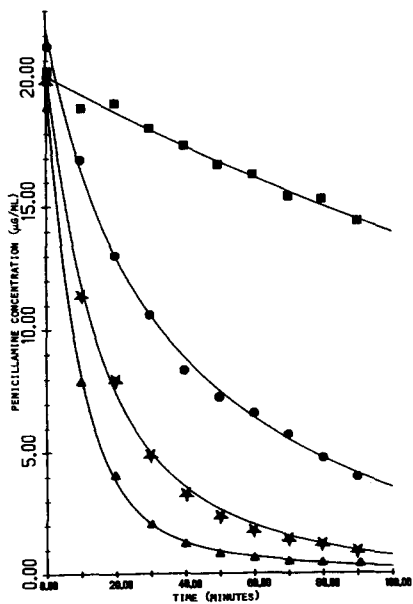circles=25°C., triangles=37°C.

FIG. 3
The Loss of Penicillamine in 1, 3,
5, and 7% Albumin Solutions at 25°C.
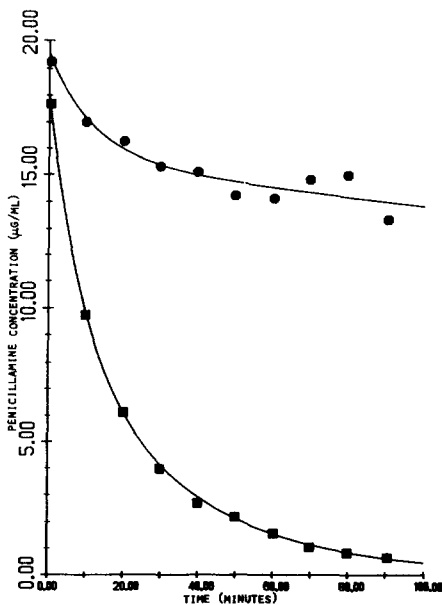squares=1%, circles=3%, stars=5%
and triangles=7%.

FIG. 4
The Loss of Penicillamine in a
5% Albumin Solution at 25°C.
squares = reduced penicillamine
circles = total penicillamine
(total = reduced + oxidized).

the same experimental conditions are shown in Table I. The control experiment results demonstrate that the loss of penicillamine is not due to a pH phenomenon alone. Aqueous solutions of penicillamine are reported to be stable in the pH range from 2-4 and thus a loss of penicillamine may be expected at physiological pH (pH=7.4). However, in the period of time considered in our experiments no penicillamine loss was observed at pH 7.4.

TABLE I

Stability of Penicillamine[1] in the Solutions Used
to Dilute the 25% Normal Human Serum Albumin

Time minutes	EDTA[2] Solution 1 g./L.		Imidazole[3] Buffer pH = 7.4	
	Penicillamine Concentration µg./ml.	Percent of Initial	Penicillamine Concentration µg./ml.	Percent of Initial
1	19.5		18.6	
10	19.4	99.5	19.0	102.2
20	19.5	100.0	19.2	103.2
30	19.5	100.0	19.2	103.2
40	19.1	97.9	19.2	103.2
50	19.2	98.5	19.0	102.2
60			18.9	101.6
70			19.0	102.2
80			18.7	100.5
90			18.2	97.8

1) A concentrated penicillamine solution was added to the test solution at time zero to produce an approximate initial penicillamine concentration of 19.0 µg./ml.
2) Ethylene diamine tetracetic acid disodium salt solution. Previous investigators (see reference 21) have also demonstrated the stability of penicillamine in EDTA solutions. The temperature was at ambient temperature (approximately 25°C.).
3) The imidazole buffer contained 4.3 g. imidazole, 250 mg. EDTA, 225 ml. HCl, q.s. to 250 ml. with deionized water. The temperature was controlled at 25°C.

The rate of loss was shown to be dependent on the concentration of albumin. Figure 3 shows the loss of penicillamine that occurred when penicillamine was added to a 1, 3, 5, or 7% solution of albumin.

The loss of penicillamine may be due to more than one reaction. The results of the study of penicillamine in a 5% albumin solution and in anticoagulated whole blood are shown in Figures 4 and 5. Not all of the original amount of penicillamine could be regenerated using the electrolysis reductive procedure described in the methods. These results indicate that perhaps more than one product is being formed from the loss of penicillamine, a product that can be regenerated (ie. disulfides) and a product that cannot.

Figures 6, 7, and 8 show the log linear results of the initial rate studies of the loss of penicillamine in a 5% solution of albumin at three different initial concentrations and at four temperatures. The two lower concentrations
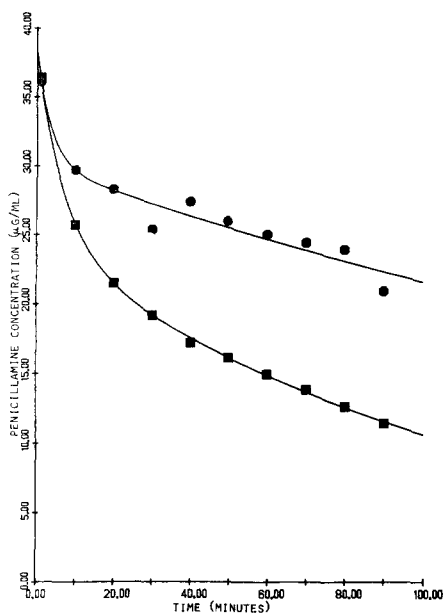
FIG. 5

The Loss of Penicillamine in EDTA
Anticoagulated Whole Blood at 25°C.
squares = reduced penicillamine
circles = total penicillamine
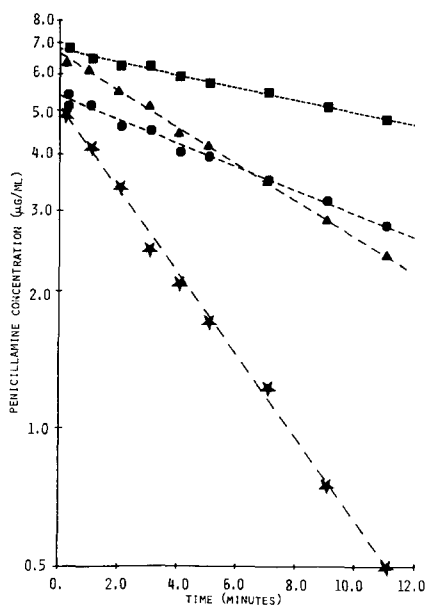(total = reduced + oxidized).

FIG. 6

The Initial Loss of Penicillamine in
a 5% Albumin Solution. (initial
concentration of 6.0 µg./ml.)
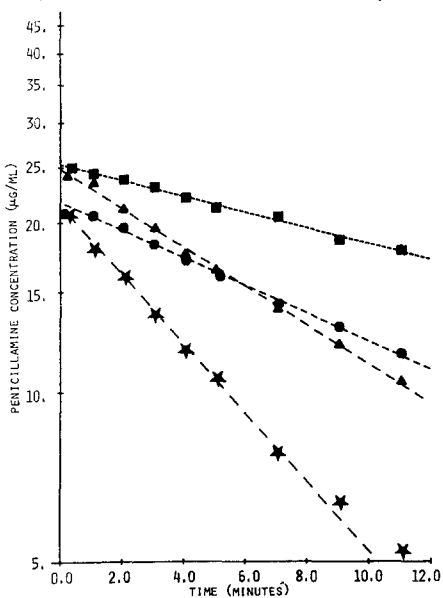squares=10°C., circles=25°C.,
triangles=37°C., stars=55°C.

FIG. 7

The Initial Loss of Penicillamine in
a 5% Albumin Solution. (initial
concentration of 23. µg./ml.)
squares=10°C., circles=25°C.,
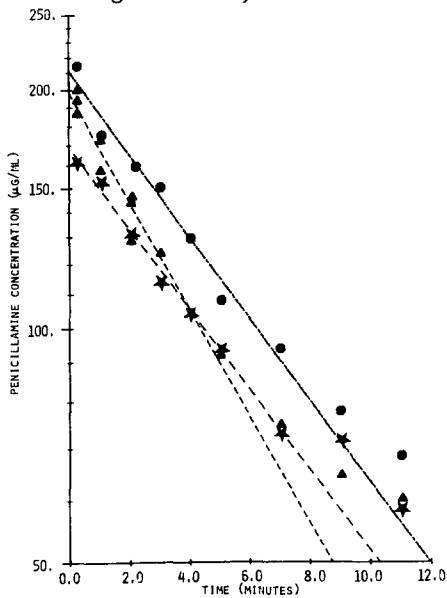triangles=37°C., stars=55°C.

FIG. 8

The Initial Loss of Penicillamine in
a 5% Albumin Solution. (initial
concentration of 200. µg./ml.)
circles=25°C., triangles=37°C.,
stars=55°C.

of penicillamine showed a temperature dependency of the rates and the Arrhenius plots for these data are shown in Figure 9. However, the highest concentration of penicillamine (200 μg./ml.) represents a 10 fold excess in penicillamine and the data are not temperature dependent. A summary of the observed rate constants for these experiments is given in Table II. The activation energies for the 6 and 23 μg./ml. initial concentration experiments were 3.81 and 3.00 Kcal./mole respectively.

TABLE II

Initial Rates of Penicillamine Loss in a 5% Albumin Solution[1]

Temperature °C.	K_{obs} (minutes^{-1})		
	Penicillamine Initial Concentration[2]		
	6 μg./ml.	23 μg./ml.	200 μg./ml.
10	0.0325	0.0326	
25	0.0602	0.0570	0.121
37	0.0933	0.0796	0.158
55	0.209	0.142	0.119

1) A 5% albumin solution corresponding to a 7.5×10^{-4} M. solution (molecular weight of albumin 66500 g./mole. see reference 22).

2) The approximate initial concentrations of penicillamine (6, 23, and 200 μg./ml.) correspond to 4.02×10^{-5} M., 1.54×10^{-4} M., and 1.34×10^{-3} M.
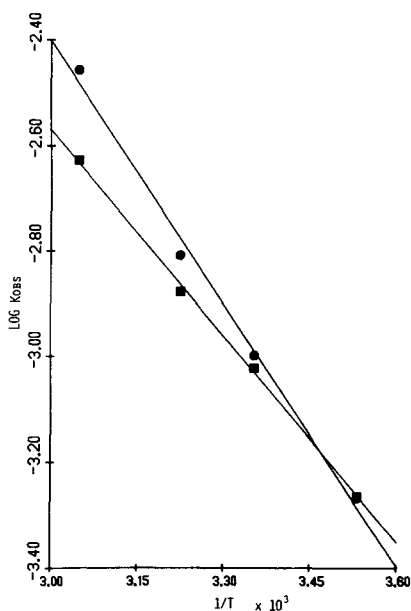

FIG. 9

Arrhenius Plots for Penicillamine Loss in a 5% Albumin Solution. circles= 6 μg./ml. squares= 23 μg./ml. initial concentrations.

The lack of a temperature dependency at the highest concentration studied indicates that the reaction mechanism may be occurring at a "specific site" on the albumin molecule. Due to the 10 fold excess of penicillamine this "specific site" would always be occupied and thus the reaction would proceed at a constant rate independent of the temperature. Studies of albumin (22) have indicated that there is only one free sulfhydryl group in the whole molecule of albumin resulting from a cysteine residual. This or some other "specific site" may be responsible for the loss of penicillamine observed.

Discussion

The loss of penicillamine in biological samples such as plasma and whole blood presents a major obstacle for the successful completion of a pharmacokinetic study of this drug. It is essential that the analytical method used to quantify the concentration of a drug measures the actual concentration of the sample as it was obtained from the subject or animal of study. The rapid disappearance of penicillamine in biological media demonstrated by the results of this paper confirm the necessity of acting quickly to stabilize the penicillamine present in a biological sample.

Previous investigators have also suggested the problem of penicillamine loss. Russell et al. (23) stated that plasma samples must be assayed as soon as they are obtained. This approach, however, limits the feasibility of most pharmacokinetic studies. Mann and Mitchell (24) stressed the necessity of using EDTA as an anticoagulant. However, the results shown in Figure 5 confirm that penicillamine is lost even in blood where EDTA was used as the anticoagulant.

TABLE III

Stability of Penicillamine[1] in a Protein Free Supernatant
of Hemolyzed Whole Blood and an EDTA Solution.

Time days	Whole Blood Supernatant[2]			EDTA Solution[3]		
	Penicillamine Conc. μg./ml.		(Percent of Initial)	Penicillamine Conc. μg./ml.		(Percent of Initial)
Initial	2.17	6.30	10.4	2.00	5.96	10.7
1	1.90 (87.6)	6.22 (98.7)	9.84 (94.6)	1.95 (97.5)	5.77 (96.8)	10.2 (95.3)
2	1.74 (80.2)	5.76 (91.4)	9.64 (92.7)	1.94 (97.0)	5.84 (98.0)	10.6 (99.0)
8	1.81 (83.4)	5.31 (84.3)	9.33 (89.7)	1.93 (96.5)	5.76 (96.6)	10.5 (98.1)

1) A concentrated solution of penicillamine was added to the whole blood supernatant or the control EDTA solutions to produce an approximate initial concentration of 1.97, 5.80 or 9.87 μg./ml. Samples were refrigerated at 4°C.

2) A protein free hemolyzed whole blood supernatant was produced by the addition of 2.0 ml. of whole blood to 2.0 ml of EDTA solution (1 g./L.) followed by the addition of 1.0 ml. of metaphosphoric acid solution (500 g./L.). After mixing and centrifugation the supernatant was decanted and filtered through a membrane filter (0.45 μ.).

3) The EDTA solution (1 g./L.) was used as a control for this experiment. Previous investigators have shown the excellent stability of reduced penicillamne in EDTA solutions (see reference 21).

As demonstrated by the results presented in this paper, the protein content particularly the albumin of the samples plays a key role in the loss of penicillamine. If blood or plasma samples are treated immediately to precipitate the proteins and lower the pH the loss of penicillamine will be minimized.

Tables III and IV show the results of a study of the stability of penicillamine in the protein free supernatant obtained from whole blood. The loss of penicillamine was minimal in these studies over a period of 6-8 days. Recovery studies of penicillamine added to whole blood was 88.2, 93.7, 85.7, and 87.7 percent.

TABLE IV

Stability of Penicillamine[1] in a Protein Free Supernatant of Hemolyzed Whole Blood Solution and an EDTA Solution

Time days	Whole Blood Supernatant[2]				EDTA Solution[3]			
	Penicillamine Conc. µg./ml.		Percent of Theoretical		Penicillamine Conc. µg./ml.		Percent of Theoretical	
Initial	4.64	14.8	92.9	98.4	4.75	15.0	95.0	100.
1	4.75	14.8	95.2	98.4	5.03	15.3	101.	102.
2	5.05	15.1	101.	101.	4.93	15.3	98.8	102.
3	4.82	14.9	96.5	99.7	4.74	15.0	95.0	100.
6	4.46	14.0	89.5	93.6	5.00	15.0	100.	100.
7	4.43	13.9	88.8	92.8	5.06	15.0	101.	100.

1) A concentrated penicillamine solution was added to the supernatant or control solution to produce an initial concentration of 5.00 µg./ml and 15.0 µg./ml. The samples were refrigerated at 4°C.
2) The protein free hemolyzed blood supernatant was produced by adding 2.0 ml. of whole blood to 7.0 ml. of EDTA (200 mg./L.) solution. After 1 minute 1.0 ml. of metaphosphoric acid (500 g./L.) solution was added. After 5 minutes the sample was centrifuged for 10 minutes at 2500 rpm. The supernatant was decanted and filtered through a membrane filter (0.45 µ.).
3) An EDTA (1 g./L.) solution was used as a control. Penicillamine has excellent stability in EDTA solutions (see reference 21).

The studies reported here are for the _in vitro_ initial rate of loss of penicillamine. However, loss of penicillamine by similar reactions probably also occurs _in vivo_. Our results indicate that the rate of reaction is reduced with time. Thus the impact on pharmacokinetic samples collected minutes to hours after the dose of penicillamine probably will be less that the initial rate of loss demonstrated by our results. Nevertheless, it is important that all samples be deproteinated as soon an possible after they are collected from the subject or animal being studied. The whole blood treatment procedure used in the above studies can generally be completed in less that two minutes.

In vitro distribution studies of penicillamine in whole blood indicate that most of the penicillamine is found in the extracellular plasma compartment.

Thus when the cell lysis occurs in the whole blood procedure, a "dilution" of the sample results beyond that caused by the addition of the EDTA diluent alone. In addition, the procedure for the treatment of whole blood samples results in a greater dilution of the actual analytical samples than is necessary to process an equivalent plasma sample (see methods). These factors coupled together mean that the analytical sample resulting from the whole blood procedure is less concentrated than the analytical sample obtained from plasma of the same blood sample. Thus as the limit of sensitivity of the analytical assay is approached, plasma samples can be assayed for a longer period of time because of their relative higher concentration. Due to this advantage, plasma samples may be more appropriate for pharmacokinetic studies of penicillamine.

To prevent the excessive loss of penicillamine, however, the plasma must be separated from the whole blood and deproteinated as quickly as possible. For this purpose we employ a micro centrifuge (15000 rpm - 30 sec.) to separate heparinized whole blood samples into plasma and packed cells. The plasma is then treated as described in the methods above. A recovery study of penicillamine added to normal plasma yielded from 103 to 109 percent. Using this methodology the blood samples can generally be processed (plasma separated and proteins precipitated) in less than two minutes.

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