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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PENICILLAMINE IN WHOLE BLOOD, PLASMA, AND URINE

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SUMMARY

A high-performance liquid chromatographic method for the determination of penicillamine in plasma, whole blood, and urine samples is described. The method uses a commercially available electrochemical detector at a potential of +0.1 V versus the Ag/AgCl reference electrode. This method is selective and sensitive for sulfhydryl compounds. The chromatography separates penicillamine from other endogenous sulfhydryl compounds with a limit of detection for penicillamine in biological samples of ca. 10^{-7} *M*.

INTRODUCTION

D-Penicillamine has been used as a medicinal agent since 1954 when it was first used for the treatment of hepatolenticular degeneration [1]. More recently it has also been used in the treatment of cystinuria [2] and rheumatoid arthritis [3]. A recent review of penicillamine [4] lists many other possible uses for this compound.

Since penicillamine has been shown to be effective for the treatment of rheumatoid arthritis [5], a great interest in research on this therapeutic agent has been stimulated due to the high incidence and debilitating nature of this diease. However, accurate pharmacokinetic studies of penicillamine and other areas of penicillamine research have been restrained due to the lack of a sufficiently sensitive and specific assay for penicillamine. Most of the pharmacokinetic studies done in the past have employed radiolabeled penicillamine

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[6—11]. The results of these studies are inappropriate for the description of penicillamine pharmacokinetics since the results represent both metabolized and parent drug.

A number of analytical methods including colorimetry [12, 13], gas chromatography [14] and radioimmunoassay [15, 16], have been developed for penicillamine. These methods either require complex sample manipulation during which penicillamine degradation may occur or are not specific for penicillamine. Thus, these methods are inappropriate for pharmacokinetic sample analysis.

The recent development of a high-performance liquid chromatographic (HPLC) method for the determination of penicillamine [17, 18] using electrochemical detection has provided a simple, specific, and sensitive assay for the investigation of penicillamine. We have modified this HPLC method and are using it in our laboratory for the analysis of penicillamine pharmacokinetic samples. The details of the modified method are reported in this paper.

MATERIALS AND METHODS

HPLC apparatus

Cationic ion-exchange chromatography was used to separate penicillamine from the other electroactive components in the analytical samples. Two columns (5 × 0.41 cm and 30 × 0.41 cm) packed with Zipax SCX (strong cation-exchange, silica microbead coated, spherical glass beads, 30 μm; DuPont; Wilmington, DE, U.S.A.) were used as guard and analytical column, respectively. A citric acid—dibasic sodium phosphate buffer (0.03 *M* and 0.01 *M*, respectively) was pumped through the columns using a Milton Roy Model 396 minipump (Riviera Beach, FL, U.S.A.) at a flow-rate of 2.5 ml/min. Prior to use, the citric acid—phosphate buffer was deoxygenated by vigorously bubbling nitrogen through the buffer solution for 20 min. Then during use, nitrogen was continuously passed over the top of the buffer solution and the PTFE tubing leading from the buffer reservoir to the pump. Deoxygenation of the buffer was necessary to prevent excessively high background currents caused by the presence of dissolved oxygen. The analytical samples and standards of penicillamine were injected onto the column using a 20-μl loop valve (Model 7010; Rheodyne, Berkeley, CA, U.S.A.).

The electrochemical detector used for this analysis was the LC-4 Amperometric Controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The detector used was a thin-layer electrochemical cell (Model TL-6A; Bioanalytical Systems) which consists of a mercury/gold amalgam working electrode, a glassy carbon auxiliary electrode, and a Ag/AgCl reference electrode. A Faraday cage was used to enclose the electrochemical cell and waste reservoir. The potential of the working electrode was maintained at +0.1 V versus the Ag/AgCl reference electrode. The background current was generally less than ca. 10 nA.

Sample preparation

Because of the rapid loss of penicillamine in plasma [19] and urine [17], the biological samples must be treated immediately upon collection to stabilize the amount of penicillamine present in the sample. This is accomplished by

decreasing the pH and/or precipitating the proteins in the samples.

For the treatment of plasma samples, 0.2 ml of trichloroacetic acid (20%, w/v) was added per ml of plasma. The precipitated samples were centrifuged and the supernatants were decanted. Whole blood samples were first hemolyzed by the addition of an EDTA solution (1 g/l) (1 ml per ml of whole blood sample) and then a metaphosphoric acid solution (500 g/l) was added to decrease the pH and precipitate the proteins. The urine samples were diluted (1:5 or 1:10) using a 0.4 *M* citric acid solution to buffer the pH to acidic conditions.

The resulting analytical samples from the above procedures should be refrigerated (1—4°C) until analyzed and the assays should be completed as soon as possible (1—4 days) after the collection of the samples to avoid a change in the concentration of penicillamine. The diluted urine samples, and the plasma and whole blood supernatants are injected directly into the HPLC system for analysis without further treatment.

Assay standards and procedures

Analytical standards of penicillamine were prepared at nine concentrations (75, 30, 15, 7.5, 3.0, 1.5, 0.75, 0.30, and 0.15 μg/ml) to be used in the assay procedure. The concentrations of these nine standards were chosen so that each standard will produce approximately a 3/4 full scale response when injected into the HPLC system with the sensitivity setting of the electrochemical detector set at one of the nine possible sensitivity settings used (500, 200, 100, 50, 20, 5, 2, and 1 nA/V). To make these standards, an accurate quantity of D-penicillamine (99%+ purity; Aldrich, Milwaukee, WI, U.S.A.) was weighed out (Cahn microbalance, Paramount, CA, U.S.A.) and was dissolved in an EDTA (1 g/l) solution to produce the most concentrated standard. From this concentrated standard all other standards were prepared by appropriate dilution using the EDTA (1 g/l) solution. The standards were filled into glass ampoules, purged with nitrogen, and flame sealed and refrigerated (1—4°C) until used. After opening the glass-sealed standards for use, the standards were stored in air-tight test-tubes, used for one week, and then discarded. Excellent stability for penicillamine solutions prepared and stored as above has been reported by others [17, 20]. Our results [19] and experience also confirm that no significant change in the concentrations of the standards occurred over a storage period of 3—6 months.

A 20-μl aliquot of each unknown analytical sample is injected via the loop valve into the HPLC system followed immediately by a 20-μl aliquot of a penicillamine standard. After the unknown analytical sample is injected and a suitable response is recorded with the electrochemical detector set at one of the nine sensitivity settings, the corresponding standards for that sensitivity setting are injected. The concentration of the unknown analytical sample is determined by multiplying the ratio of the observed peak heights (unknown sample:standard) times the concentration of the standard used. The concentration of the unknown analytical sample must then be corrected for dilution caused by the sample preparation procedure to give an estimate of the concentration of the actual biological sample. To minimize errors, the ratio of the peak heights of the unknown analytical sample to that of the penicillamine standard should be close to unity.

RESULTS

The chromatography of penicillamine and other similar sulfhydryl compounds using Zipax SCX has been well described by Rabenstein and coworkers [17, 18, 21—24]. As shown by these workers, the retention of penicillamine can be increased or decreased by decreasing or increasing, respectively, the pH of the eluting buffer. Table I shows the different compositions of citric acid—phosphate buffer which may be used for the chromatography of penicillamine. The approximate pH of the buffer and the relative effect of the buffers on the retention times of penicillamine are also indicated. Considerable differences between individual columns have been noted during our experience. Maintaining a constant flow-rate, it was necessary to use buffers 5 and 7 to achieve comparable retention times on two different columns.

Fig. 1 shows chromatograms of the separation of penicillamine in whole blood, plasma and urine samples. Each unknown sample that is injected into the HPLC system is followed by an injection of a penicillamine standard. Fig. 2 shows a chromatogram of penicillamine in an EDTA solution. This chromatogram demonstrates the potential sensitivity of this method as the peak shown represents 10^{-9} *M* penicillamine.

The recoveries of penicillamine added to plasma and urine samples of a normal volunteer are shown in Table II. Each of the different concentrations studied were done in triplicate. The theoretical concentrations of penicillamine were based upon the amount of penicillamine added in a concentrated solution form to the normal human plasma or diluted urine and the total volume of the biological sample after this addition. The volume of concentrated penicillamine solution was always less than 3.5% of the total sample volume.

For the recovery studies, the urine was diluted 1:5 with 0.4 *M* citric acid solution prior to the addition of the concentrated penicillamine solution. The plasma samples were treated immediately after the addition of the concen-

TABLE I

RESULTING pH AND RELATIVE PENCILLAMINE RETENTION OF CITRIC ACID—SODIUM DIBASIC PHOSPHATE BUFFERS OF VARIOUS COMPOSITION

Buffer No.	Buffer Composition (*M*)		pH	Relative* penicillamine retention
	Citric acid	Na_2HPO_3		
1	0.0400	0.0000	2.66	
2	0.0375	0.0025	2.72	
3	0.0350	0.0050	2.84	
4	0.0325	0.0075	2.95	
5	0.0300	0.0100	3.09	
6	0.0275	0.0125	3.25	decrease
7	0.0250	0.0150	3.37	
8	0.0225	0.0175	3.58	
9	0.0200	0.0200	3.87	

*The relative retention of penicillamine when penicillamine is chromatographed using the indicated buffer system and a Zipax SCX column.

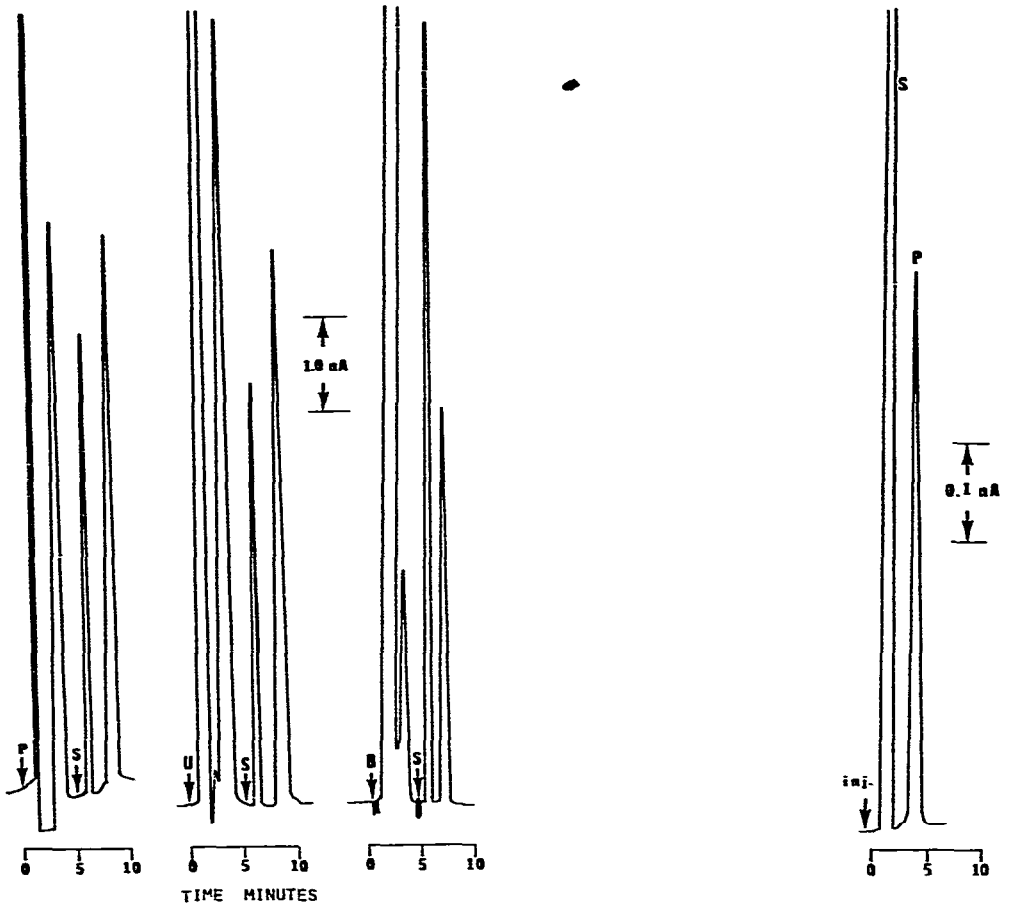

Fig. 1. Separation of penicillamine in whole blood, plasma, and urine samples. Injection points: P = plasma; U = urine; B = whole blood and S = penicillamine standard.

Fig. 2. A chromatogram of 10^{-3} *M* penicillamine in a solution of EDTA (1 g/l). S = solvent front, P = penicillamine peak, and inj. = sample injection.

trated penicillamine solution by the procedures outlined in Materials and methods section. The poor recovery of penicillamine from the plasma samples at the two lowest concentrations is due to the initial rate of loss of penicillamine as observed in the results of our recent publication [19]. Thus for statistical analysis these data were omitted.

The loss of penicillamine associated with the presence of plasma proteins probably also occurs in vivo. Therefore at the time of sampling the reaction rate will be much less than the initial rate observed in vitro as is the case in these recovery studies. Our preliminary results indicate that very little change in the penicillamine concentration occurs if the samples are treated immediately upon collection by the procedures outlined in Materials and methods section.

TABLE II

RECOVERY OF PENICILLAMINE FROM PLASMA AND URINE

Theoretical Conc. (μg/ml)	Normal plasma		Normal urine (diluted)	
	Mean percent recovered*	C.V.** (%)	Mean percent recovered*	C.V.** (%)
72.4	100	5.25	108	2.23
36.8	103	2.25	107	2.69
18.6	107	3.19	112	1.60
7.24	99.4	2.96	108	1.21
3.68	101	2.20	110	1.93
1.86	102	5.94	113	2.28
0.724	94.8	4.32	106	1.37
0.368	82.5***	2.55	98.9	2.14
0.186	77.4***	7.30	103	0.70
Grand§	101.0	3.68	107.0	4.25

*The mean recovery from three samples for each concentration using individually prepared plasma or diluted urine specimens and reported as a percentage of the theoretical concentration.
**The coefficient of variation of the three observed samples.
***These data were omitted from the grand mean because of a significant effect of the initial rate of loss of penicillamine (see text).
§ The grand mean and coefficient of variation of all the observed samples.

DISCUSSION

The HPLC method described in this paper utilized commercially available equipment unlike the previously published method [17] which required a homemade thin-layer electrochemical cell. Our method differs also in that a pre-established calibration curve for pencillamine is not necessary; rather the unknowns are quantitated by injecting a standard of penicillamine of similar concentration immediately after the unknown. By a comparison of the peak heights of the unknown and standard, the concentration of the unknown sample can be calculated.

The above method for quantitation of the unknown samples was used instead of the usual internal standard technique because a suitable internal standard for penicillamine could not be found. Due to the specificity of the detector, the choice of an internal standard is primarily limited to other sulfhydryl compounds, many of which are endogenous.

However, many of the classical reasons for using an internal standard were not necessary for this assay. The sample preparation does not require an extraction procedure and only a dilution of the actual biological sample is necessary. The samples are injected onto the HPLC column using a precise loop valve which reduces the injection volume error to a level below that of the error of the overall assay [25]. Thus for this assay, an internal standard is not necessary to quantitate the efficiency of an extraction process or the amount of sample injected.

The use of the standard injection after the sample vastly reduced the error in the estimation of the amount of penicillamine in an unknown sample because the response of the detector was not always consistent. Due to a deterioration of the surface of the mercury/gold amalgam working electrode, the response of the electrode changes over a period of time and the electrode must be resurfaced frequently. The frequency of resurfacing the working electrode's surface is dependent upon the types of samples analyzed and the conditions used. A newly resurfaced electrode may operate efficiently for only several minutes or for several days. The surface of the electrode should be resurfaced at a minimum of once every five days, however, following continual usage to ensure an adequate response. During the course of a particular HPLC run, the response of the electrode is consistent for a period of time, however, the response slowly changes with continued use. Thus, comparing the results of an unknown sample to a calibration curve made earlier in the same run or during another run would lead to erroneous results. But a comparison of the sample to a standard injected at the same time leads to a minimal error resulting from the changes in the response of the detector.

The assay reported in this paper is specific for reduced penicillamine. The metabolites of penicillamine as shown in Fig. 3 include penicillamine disulfide, penicillamine cysteine disulfide, and S-methyl-pencillamine [26]. These metabolites are not detected in this assay because of the specificity of the electrochemical detector. At the conditions employed by the detector, only reduced sulfhydryl compounds will be detected. The endogenous sulfhydryl compounds, cysteine, glutathione, homocysteine, and ergothionine, are separated from penicillamine by chromatography, which has been described by other investigators [17, 21—24].

Plasma and urine samples from five normal volunteers and five rheumatoid arthritis patients were injected into the HPLC system before and after the addition of exogenous penicillamine to check for any endogenous peaks that would interfere with the peak for pencillamine. The rheumatoid arthritis patients were not taking penicillamine but were using other medications including aurothioglucose, aurothiomalate, aspirin, prednisone, sulindac, hydroxychloroquine sulfate, aminophylline, iron, and multiple vitamin products. None of the samples from the volunteers or rheumatoid arthritis

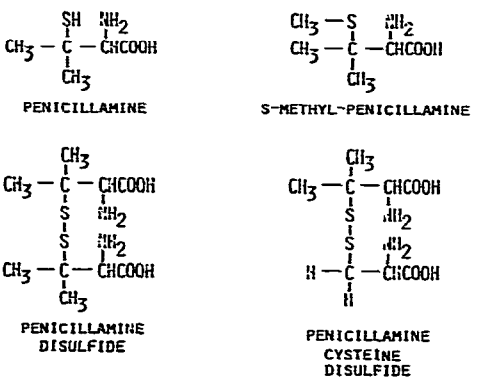

Fig. 3. Structure of penicillamine and related compounds.

patients displayed any peaks that would interfere with the analysis of penicillamine.

The HPLC method reported in this paper is suitable for the determination of unchanged penicillamine in biological samples for pharmacokinetic analysis or other research applications. The results of the application of this method to the samples of a pharmacokinetic study of penicillamine in animals and man in the authors' laboratory are being prepared for publication. The limits of detection of penicillamine in biological samples are $5 \cdot 10^{-7}$ *M* for plasma and undiluted urine samples and $3 \cdot 10^{-6}$ *M* for whole blood samples due to the additional dilution of whole blood samples during processing.

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