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# The determination of D-penicillamine and its disulphide in plasma by reversed-phase ion-pair high-performance liquid chromatography

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**Abstract**: Reversed-phase ion-pair conditions are used for the determination of D-penicillamine and penicillamine disulphide. Two chromatographic systems were employed, one for penicillamine and the other for penicillamine disulphide. The procedures permit the determination of total penicillamine (protein-bound, free and as disulphides) in whole plasma, and total penicillamine (free and as disulphides) in plasma ultrafiltrate, using an incubation step in the presence of dithiothreitol. Free penicillamine and penicillamine disulphide may be determined independently by direct injection of plasma ultrafiltrate. Both solutes may be measured at an on-column sensitivity of 10 ng, utilizing an electrochemical detector based on a glassy carbon electrode.

**Keywords**: *D-Penicillamine analysis; penicillamine disulphide analysis; high-performance liquid chromatography; reversed-phase chromatography; ion-pair chromatography.*

## Introduction

D-Penicillamine has been used in the last twenty-five years for the treatment of a variety of diseases, including Wilson's disease [1], cystinuria [2], heavy metal poisoning [3], chronic active hepatitis [4], primary biliary cirrhosis [5] and rheumatoid arthritis [6]. Pharmacokinetic studies have been hampered by the lack of sensitive analytical procedures routinely applicable to plasma samples, although methods employing colorimetry [7, 8], amino-acid analysis [9, 10], gas–liquid chromatography [11, 12] and radioimmunoassay [13] have been described. The development of a high-performance liquid chromatographic (HPLC) method [14–16] utilizing an ion-exchange column and a mercury-based electrochemical detector has provided a specific and sensitive assay for penicillamine in plasma. Bergstrom *et al.* [17, 18] simplified this method by using a commercially-available gold amalgam working electrode specific for the detection of sulphydryl compounds, and applied it to a pharmacokinetic study of penicillamine. This paper describes the direct measurement of penicillamine and its disulphide metabolite (PSSP), using the less specific glassy carbon electrode detector, coupled with ion-pair chromatography for good selectivity and flexibility of solute retention [19]. This work

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continues our earlier study of analytical methods for drugs such as non-steroidal anti-inflammatory agents [20], employed in the treatment of rheumatic diseases.

## Experimental

### *Chemicals*

Penicillamine (PSH), penicillamine disulphide (PSSP) and dithiothreitol (DTT) were obtained from Sigma, Poole, UK; disodium hydrogen phosphate, ethylenediaminetetra-acetic acid disodium salt (EDTA), citric acid, sodium dodecylsulphate (SDS) from BDH, Poole, UK; dodecyltrimethylammonium bromide from Cambrian Chemicals, Croydon, UK; *p*-methoxyphenylacetic acid from Koch-Light, Colnbrook, UK; *o*-aminobenzoic acid from Aldrich, Gillingham, UK; and methanol (HPLC grade) from Fisons, Loughborough, UK. All chemicals were used as received. Deionized double-distilled water was used throughout.

### *Instrumentation*

Separations were performed on columns of Spherisorb Amino (25 × 0.5 cm) for penicillamine and LiChrosorb RP8 (15 × 0.5 cm) for penicillamine disulphide. Both columns were packed with 5 μm material by upward displacement using 20% v/v methanol in chloroform. Columns were stabilized with the appropriate mobile phase containing the pairing-ion at 1 ml/min for 16 h. An in-line column (5 × 0.5 cm) containing LiChroPrep RP-18 (25–40 μm), and situated prior to the injection valve, was used in each case to protect the analytical column from adverse effects of the mobile phase. A Du-Pont degasser/solvent reservoir unit was used to degas the mobile phase (15 min) and maintain this condition for at least 24 h. Analytical samples were introduced on to the column using a 20 or 100 μl loop valve (Rheodyne 7125, Jones Chromatography, Llanbradach, UK). The temperature of the mobile phase, column and injector was controlled by immersion in a water bath (Gallenkamp type 400–010) at 25° or 35°C. The pump was a Constametric III (Jones Chromatography) and the LC-4 electrochemical detector (Bioanalytical Systems, Anachem, Luton, UK) was fitted with a thin-layer cell having a glassy carbon working electrode (TL-5) and a Ag–AgCl reference electrode (RE-1).

### *Procedures for penicillamine*

(i) *Calibrations.* A stock solution of penicillamine in mobile phase containing EDTA ($2.7 \times 10^{-3}$M) was used to prepare six dilutions in the range $7 \times 10^{-4}$M to $4 \times 10^{-6}$M in mobile phase containing *p*-methoxyphenylacetic acid ($6 \times 10^{-6}$M) as internal standard. An identical calibration was carried out for penicillamine added to plasma. The required amount of stock penicillamine solution was aded to 1.00 ml plasma, followed by 0.36 ml dithiothreitol solution (0.065 M); the volume was made up to 3 ml with disodium hydrogen phosphate (0.05 M). Incubation at 60°C for 4 h was followed by filtration through a Centriflo CF50A filter (Amicon, Woking, UK) at 3000 rpm for 15 min. The ultrafiltrate (0.3 ml) was made up to 1.00 ml with mobile phase containing internal standard to produce the same range of penicillamine concentrations as before. All solutions were injected in triplicate.

(ii) *Total PSH in plasma.* Plasma samples (1 ml) were treated with 0.24 ml dithiothreitol solution (0.065 M), made up to 2 ml with disodium hydrogen phosphate

(0.05 M) and incubated at 60°C for 3 h. Following filtration through a Centriflo CF50A filter, the ultrafiltrate (0.3 ml) was mixed with an equal volume of mobile phase containing internal standard and assayed. Alternatively, the method employed by others [15, 17] was used to prepare a protein-free haemolysed blood supernatant: whole blood (2 ml) was added to EDTA solution (7 ml, $5.4 \times 10^{-4}$M) and after 1 min metaphosphoric acid solution (1 ml, 5.6 M) was added. After 5 min the sample was centrifuged for 10 min at 2500 rpm. The supernatant was decanted and filtered through a membrane filter (0.45 μm). Plasma samples (1 ml) with 3.5 ml EDTA solution ($5.4 \times 10^{-4}$M) containing internal standard ($6 \times 10^{-6}$M) and 0.5 ml metaphosphoric acid solution (5.6 M) were used in this work.

(iii) *Free PSH in plasma.* Ultrafiltrates of plasma samples were obtained as above, mixed with an equal volume of mobile phase containing internal standard, and assayed.

(iv) *Total PSH in plasma ultrafiltrate.* Ultrafiltrates of plasma samples were incubated as in (ii) above, diluted with an equal volume of mobile phase containing internal standard, and assayed.

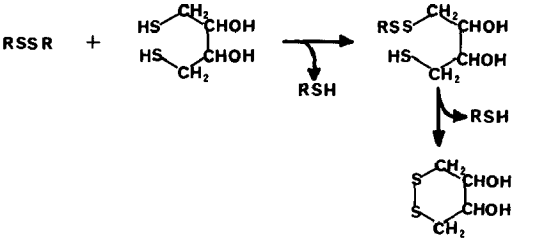
*Procedure for penicillamine disulphide (PSSP)*

Calibrations for penicillamine disulphide in mobile phase containing *o*-aminobenzoic acid as internal standard ($1.1 \times 10^{-6}$M) were carried out using six concentrations in the range $7 \times 10^{-4}$ to $4 \times 10^{-6}$M. An identical calibration was carried out in the presence of plasma, using 1.00 ml each of calibration solution and plasma. The ultrafiltrate of this mixture was prepared and 0.5 ml mixed with an equal volume of mobile phase containing internal standard. Ultrafiltrates (0.2 or 0.4 ml) of plasma samples were diluted with an equal volume of mobile phase containing internal standard and assayed.

The concentrations of all unknown samples were determined by comparison with two calibration solutions containing penicillamine or its disulphide, and internal standard. All solutions were injected at least in duplicate, with two unknowns being followed by two calibration solutions, in order to compensate for any gradual changes in detector sensitivity.

**Results and Discussion**

It has been shown [17] that *in vitro* concentrations of penicillamine in plasma or albumin solutions decrease rapidly with time, and that this loss is mainly, but not entirely, due to disulphide formation. The loss can be minimized by treating whole blood immediately to separate the plasma, and by deproteinizing the latter and reducing its pH. The sample should then be refrigerated (1–4°C) until analysed (1–4 days), or frozen (−21°C). The method described here employed metaphosphoric acid for treating plasma [15, 17] and produced samples compatible with the chromatographic conditions used for penicillamine determination. The suitability of dithiothreitol for the reduction of penicillamine disulphide and any other protein or non-protein disulphide form of penicillamine occurring in plasma was also investigated, since this reagent is more specific than protein precipitation with acid. Dithiothreitol (2,3-dihydroxy-1,4-dithiol-butane) was introduced by Cleland [21] as a protective agent for −SH groups. It is a highly water-soluble solid with little odour, and is capable of reducing disulphides quantitatively and maintaining thiols completely in the reduced state. The reduction reaction is:




and requires only several minutes at pH 8.0 [21]. The effectiveness of dithiothreitol (DTT) in converting penicillamine disulphide into penicillamine (PSH) in plasma was studied by comparing the PSH content of a penicillamine solution ($4 \times 10^{-6}$M) with the PSH content of a penicillamine disulphide solution ($2 \times 10^{-6}$M) when both were incubated with dithiothreitol. Figure 1 shows that employing a DTT concentration of $7.8 \times 10^{-3}$M in the incubate (approximately 10 times the maximum molar concentration of PSH in the calibration range) gave an 89% yield of PSH after 3 h incubation at 60°C. This yield was reproducible and stable.

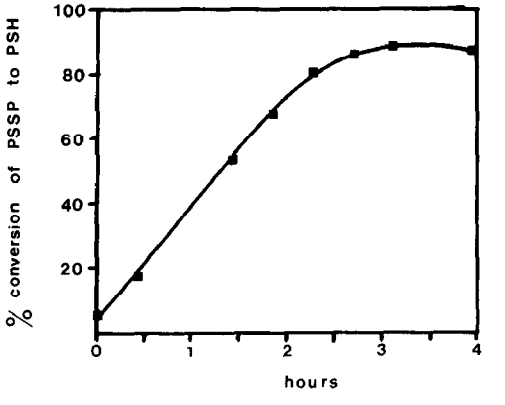



**Figure 1**
Formation of reduced penicillamine from penicillamine disulphide following incubation with dithiothreitol. For conditions, see text.

The chromatographic analysis of penicillamine and related compounds is an interesting problem that has been partially solved by using cation-exchange HPLC [14–18]. Recently, Perrett [22] has shown that, using cation-exchange column chromatography with ninhydrin detection, all the known metabolites of penicillamine present in urine may be separated in about 3 h. He employed an amino acid analyser unit in conditions similar to those described by Purdie *et al.* [10]. Cation-exchange HPLC has also been described [23] in which various organo-sulphur compounds including penicillamine were detected by post-column reaction employing a palladium (II)-calcein reagent in an air-segmented flow unit. The detection limit was about 20 ng penicillamine for spiked serum or urine, with an analytical time for serum of about 48 min. The reagent employed in the colorimetric determination of penicillamine [8] has also been applied to a post-column reaction HPLC method by Beales *et al.* [24].

The present approach was less complex, using reversed-phase ion-pair techniques coupled with the relatively robust glassy carbon electrochemical detector. Penicillamine has three ionizable functional groups (thiol, carboxylic acid and primary amine) having $pK_a$ values of approximately 10.5, 2 and 8 respectively. The thiol group is unavailable for

ion-pairing below pH 8.5, a value above the usual upper limit of pH stability for HPLC columns. The complete ionization of the carboxylic acid group above pH 4, and the complete protonation of the amino group below pH 6 ensure that PSH and PSSP are present as zwitterions within this range, and thus unavailable for ion-pairing. However, below pH 4 penicillamine will exhibit an increasing net positive charge due to progressive suppression of the carboxylic acid group so that ion-pairing with negatively charged reagents becomes possible. Conversely, the amino group is increasingly deprotonated above pH 6 whilst the carboxylic acid remains fully ionized, so that positively charged ion-pairing reagents may be used.

**Table 1**
Chromatographic conditions

| | Penicillamine | Penicillamine disulphide |
|---|---|---|
| Mobile phase | 5% v/v methanol in 0.05 M phosphate buffer containing $2.7 \times 10^{-4}$M EDTA, and $5 \times 10^{-3}$M sodium dodecyl-sulphate, pH 3.2 | 12% v/v methanol in 0.05 M phosphate buffer containing $2 \times 10^{-4}$M dodecyltrimethylammonium bromide, pH 7.8 |
| Stationary phase | Spherisorb $NH_2$, 5 μm, 25 × 0.5 cm | LiChroSorb RP8, 5 μm, 15 × 0.5 cm |
| Temperature | 25°C | 35°C |
| Flow rate | 2 ml/min | 1.8 ml/min |
| Injection volume | 100 μl | 100 μl |
| Sensitivity | 20 nA | 20 nA |
| Applied detector potential | +1.2 V | +1.08 V |
| Internal standard | *p*-Methoxyphenylacetic acid | *o*-Aminobenzoic acid |

Both acidic (pH 3.2) and weakly alkaline (pH 7.8) conditions were used (Table 1) and found to provide adequate resolution between penicillamine, its disulphide, and other related compounds. A number of column packing materials were investigated in each system and large differences in retention and peak shape obtained for different materials using the same mobile phase. For example, solute retention using the irregular-shaped material LiChrosorb RP-8 was much greater than when the spherical material Spherisorb Hexyl was used, although both materials have the same 14% carbon loading. Peak shapes using the relatively polar Spherisorb Amino material were sharper than when Spherisorb ODS was used in acidic conditions.

Investigation of the glassy carbon cell in acidic conditions for current–voltage response showed that penicillamine and cysteine gave a maximum response between +0.9 and +1.1 V, whereas the penicillamine disulphide response was still increasing at +1.4 V. Furthermore, the sensitivity to the thiols (10 ng on-column) was about 1000 times better than for the disulphide, which rendered the acidic conditions unsuitable for measurement of plasma levels of the disulphide. However, in weakly alkaline conditions, it was found that between +1.08 and +1.20 V sensitivity to the disulphide permitted 10 ng (on-column) to be quantified. Unfortunately, penicillamine is unstable in this mobile phase due to disulphide formation and gives rise to several asymmetrical peaks; hence the need for different chromatographic conditions for the separate determination of PSH and PSSP (Table 1). These conditions have been used in combination with the two techniques of (a) releasing penicillamine from disulphide bonds using dithiothreitol, and (b) the preparation of a protein-free plasma ultrafiltrate, to enable the following determinations to be made: whole plasma (total PSH), and ultrafiltrate (free PSH, total PSH, free PSSP).

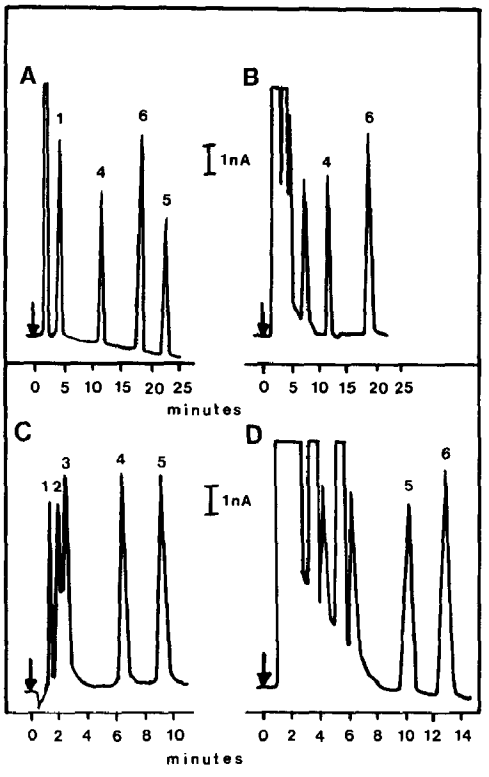



**Figure 2**
The separation of penicillamine and related compounds at pH 3.2 (A, B) and pH 7.8 (C, D). Conditions as Table 1. A and C, standard solutes in mobile phase; B, plasma processed for total penicillamine content; D, plasma ultrafiltrate diluted with mobile phase containing internal standard. 1 = cysteine; 2 = cystine; 3 = penicillamine cysteine disulphide; 4 = D-penicillamine; 5 = penicillamine disulphide; 6 = internal standard.

The calibration curve for penicillamine in the presence of plasma was linear over the range $7 \times 10^{-4}$ to $4 \times 10^{-6}$M, having the following regression values: slope, 2.245; intercept, 0.085; standard deviation of slope, 0.097. Comparison with a similar calibration prepared in the absence of plasma (slope, 2.564; intercept, 0.031; standard deviation of slope, 0.062) indicated an 87.6% recovery of penicillamine. The calibration for penicillamine disulphide in the presence of plasma was also linear over the same concentration range, having the following values: slope, 1.741; intercept, −0.050; standard deviation of slope, 0.059. Comparison with a similar calibration in the absence of plasma (slope, 1.938; intercept, −0.045; standard deviation of slope, 0.045) gave a recovery of 89.8%. These recoveries are similar to those of Bergstrom *et al.* [18] who obtained 94.8 and 77.4% from original penicillamine concentrations of 0.724 and 0.186 μg/ml respectively.

The proposed procedures have been used to determine the distribution of PSH in the plasma of five rheumatoid patients, summarized in Table 2. The results illustrate the considerable differences that occur in total PSH levels between patients, although plasma levels are clearly related to dosage. Saetre and Rabenstein [15] examined the plasma of six rheumatoid arthritic patients receiving 750 mg/day penicillamine and obtained values between 18 and 54 μM; in this work the values lie between 7 and 78 μmol/l for patients on lower dose regimens. Considering the differences in procedures and sampling involved, these figures appear to be compatible., However, the present data suggest differences in the distribution of PSH: figures for total PSH in the ultrafiltrate represent between 8 and 16% of the total PSH plasma content, indicating that between 92 and 84% of PSH is protein-bound. The data of Saetre and Rabenstein

**Table 2**
Penicillamine distribution in plasma of patients receiving penicillamine for rheumatoid arthritis

| Subject | PSH dose (mg/day) | $[PSH]_{Total}$* (μmol/l) | $[PSH]_{Total}$† | | [PSSP] | | $[PSH]_{Free}$ | |
|---|---|---|---|---|---|---|---|---|
| | | | μmol/l | % | μmol/l | % | μmol/l | % |
| 1 | 125 | 7.7 | 0.6 | 7.8 | 0.2 | 5.2 | NIL | |
| 2 | 125 | 35.7 | 3.5 | 9.8 | 1.3 | 7.3 | NIL | |
| 3 | 250 | 36.1 | 5.5 | 15.2 | 2.0 | 11.1 | -‡ | |
| 4 | 250 | 53.1 | 6.6 | 12.4 | 3.0 | 11.3 | 0.07 | 0.1 |
| 5 | 625 | 78.2 | 12.1 | 15.5 | 5.4 | 13.8 | 1.0 | 1.3 |

* Total penicillamine (protein-bound, free and disulphide metabolites) in whole plasma.
† Total penicillamine (free and disulphide metabolites) in plasma ultrafiltrate.
‡ Insufficient plasma available.

[15] suggest that only about one third of the PSH is protein-bound. This difference is probably due to the greater specificity of the reagent dithiothreitol being used to release protein-bound PSH; metaphosphoric acid requires a further electrolytic step to complete PSH release, perhaps from protein fragments as well as from the two metabolites, PSSP and penicillamine cysteine disulphide (PSSCYS). This aspect is under further study. Perrett [22] in his examination of data from various sources has suggested a figure of 80% for protein-bound PSH. Direct measurement of PSSP in this work showed it to comprise between 5 and 14% (calculated as PSH) of the total plasma PSH, whilst free PSH was either very low (1%) or undetectable. Chromatographic conditions are now being altered to measure the mixed disulphide PSSCYS. The results from the small number of patients examined here demonstrate the potential of the procedures described for a more comprehensive examination of the distribution of D-penicillamine in plasma.

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