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Note

Determination of penicillamine and other thiols by combined high-performance liquid chromatography and post-column reaction with Ellman's reagent: application to human urine

DENISE BEALES, ROSEMARY FINCH and ANDRÉ E.M. McLEAN*

Laboratory of Toxicology and Pharmacokinetics, School of Medicine, University College London, 5 University Street, London WC1E 6JJ (Great Britain)

MAURICE SMITH

DHSS Toxicology Unit, 59 St. Bartholomew's Close, London EC1A 7ED (Great Britain)

and

IAN D. WILSON

Hoechst Pharmaceuticals, Walton Manor, Milton Keynes, Buckinghamshire MK7 7AJ (Great Britain)

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Penicillamine (β,β-dimethylcysteine) is widely used in the treatment of a number of diseases including rheumatoid arthritis, Wilson's disease, cystinuria, and heavy-metal poisoning [1]. Many toxic side-effects have been noted and there is a wide variability in therapeutic effect. A simple, rapid and specific analytical method for the determination of penicillamine in biological fluids is needed to investigate these effects. Several approaches to the analysis of penicillamine have been described, including colorimetry [2], gas—liquid chromatography [3] and amino acid analyser methods [4], but few of these methods meet the desired criteria. More recently a high-performance liquid chromatographic (HPLC) method using electrochemical detection specific for thiols has been reported [5]. However, the expense of the detector, coupled with the expertise required for its operation, encouraged us to investigate alternative methods.

We now describe a method whereby, after chromatographic separation, thiols react with Ellman's reagent in a solid-bed post-column reactor to produce a coloured anion. Concurrently with our own work a sulphur-specific post-column

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reaction has been developed [6], which is also capable of detecting penicillamine.

EXPERIMENTAL

Apparatus

The analytical system employed in this study is illustrated in Fig. 1. Mobile phase was delivered using a Laboratory Data Control (Stone, Great Britain) Constametric III pump at 1 ml min^{-1}. Ellman's reagent was pumped into the post-column reactor by a Waters Assoc. (Northwick, Great Britain) Model 6000A pump at 0.5 ml min^{-1}. Samples were injected on to the column via a Waters Assoc. Model U6K universal injector. Changes in the absorbance of the eluent from the post-column reactor were monitored with a Varian (Walton-on-Thames, Great Britain) Vari-Chrom, variable-wavelength detector at 412 nm.

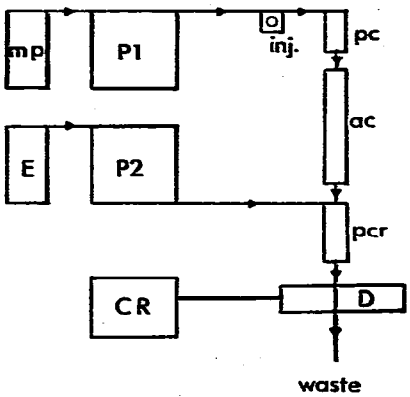


Fig. 1. Schematic diagram showing the system used in these experiments. mp = mobile phase, E = Ellman's reagent, P1 and P2 = pumps, inj. = injector, pc = precolumn, ac = analytical column, pcr = post-column reactor, D = detector, CR = chart recorder.

Chromatography was performed on a 4.5-cm precolumn connected to a 10-cm analytical column (4.6 mm I.D., stainless steel). Columns were slurry packed in methanol with 5-μm ODS Hypersil (Magnus Scientific, Sandbach, Great Britain) using a Magnus P6000 slurry packing unit.

The post-column reactor was constructed as described in ref. 7, and consisted of a stainless-steel tube, 15 cm × 2 mm I.D., filled with 40-μm glass beads (Magnus Scientific).

Post-column reduction of disulphides was performed on a column of dihydrolipoamide beads (Pierce and Warriner, Chester, Great Britain) packed into a stainless-steel column, 15 cm × 4.6 mm I.D., which is inserted between the analytical column and the post-column reactor. For best results beads were reduced with mercaptoethanol according to the manufacturer's instructions, either before use, or on oxidation.

Chemicals

All chemicals including thiols were of the purest grades available and were used without further pretreatment. Solutions of penicillamine (Sigma, Poole,

Great Britain) and other thiols used for preparing standard curves were prepared daily in mobile phase.

The mobile phase consisted of 1 g of heptanesulphonic acid (HSA; Magnus Scientific) and 150 mg of EDTA (sodium salt); (BDH, Poole, Great Britain) dissolved in 1 litre of distilled water at pH 4.

Ellman's reagent consisted of 200 mg of 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma) and 10 g of tripotassium citrate made up in 100 ml of 0.25 m*M* phosphate buffer (pH 7.4). This stock solution was diluted ten-fold with distilled water immediately before use. Both solutions were de-gassed and filtered before use.

Penicillamine determination

Sample preparation was limited to the addition of 1—2 mg ml^{-1} EDTA to urine samples immediately after collection. Homocysteine (5 μg ml^{-1}) was added to urine samples as an internal standard. Penicillamine concentrations in unknowns were determined by comparison with a standard curve constructed using blank urine spiked with known quantities of penicillamine over the range 0—50 μg ml^{-1} and 5 μg ml^{-1} internal standard and treated as above. A 20-μl sample volume was injected onto the column.

RESULTS AND DISCUSSION

The system is illustrated diagrammatically in Fig. 1. Penicillamine is a polar, ionisable compound and to obtain retention during reversed-phase chromatography an ion-pair reagent (HSA) was added. The addition of a small quantity of EDTA to the mobile phase was necessary to prevent on-column oxidation of thiols. Following separation, the thiols were mixed with a stream of Ellman's reagent at the top of the post-column reactor. Reaction and further mixing occurred as the thiol and reagent flowed through the reactor. The narrow bore of the reactor and the small bead size ensure that band spreading is reduced to a minimum [7].

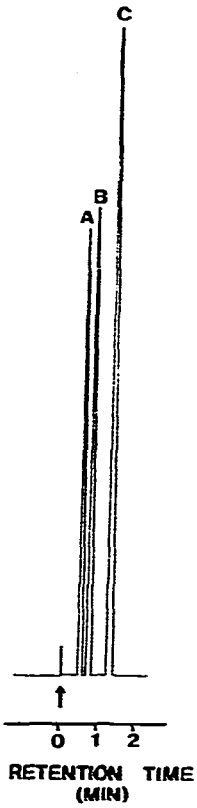
Under the conditions described the time available for reaction, before the thiol passes through the detector flow cell, is 17 sec, in which time the reaction goes to about 90% completion.

The reaction of thiols (PS^-) with Ellman's reagent (E—S—S—E) [8], shown below, produces a yellow anion (E^-) absorbing strongly at 412 nm.

$$PS^- + E—S—S—E \rightleftharpoons P—S—S—E + E^-$$

In Fig. 2 the separation and detection of a mixture of cysteine, homocysteine, and penicillamine is shown. The linear range of the system is large, allowing quantities of thiols over the range 10 ng to 150 μg (on column) to be determined.

Calibration curves for penicillamine, cysteine and glutathione over the range 0—1 μg (on column) are shown in Fig. 3. There is no response to other amino acids, or to non-SH-containing compounds, such as disulphide. The system is also insensitive to the gross contaminants present in biological fluids. Calibration curves from distilled water and urine, using homocysteine as internal standard, were identical.



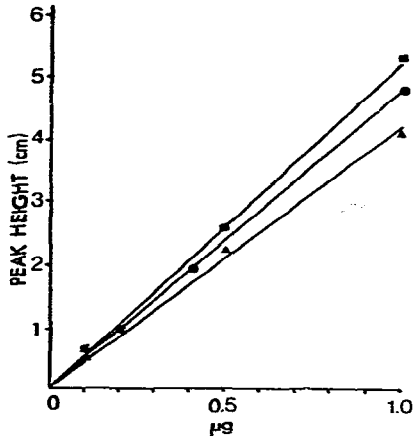


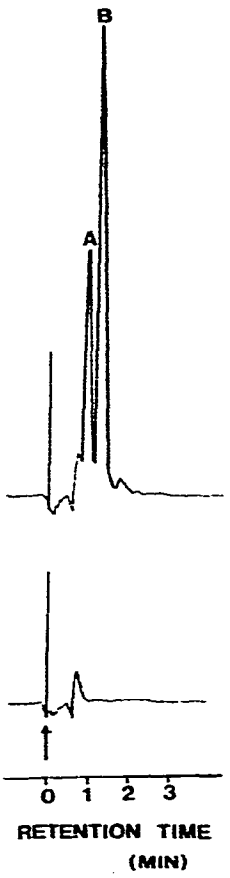
Fig. 2. Separation of a mixture of 6 μg each of cysteine (A), homocysteine (B) and penicillamine (C). Absorbance of cysteine = 1.08, homocysteine = 1.13, penicillamine = 1.56.

Fig. 3. Calibration curves for gluthathione (▪), penicillamine (●) and cysteine (▲) over the range 0—1 μg. Each point represents the average value of at least two determinations.

We have applied this method to the analysis of a number of urine samples obtained from patients treated with penicillamine (Fig. 4). For these preliminary experiments we used homocysteine as an internal standard. In future work we hope to use a penicillamine analogue. A typical HPLC trace from the urine of a patient treated with penicillamine (250 mg/day) is given in Fig. 4. Penicillamine is readily detected and we have observed levels of drug up to 60 μg ml^{-1} in patients taking between 250 and 750 mg/day.

Penicillamine solutions in HSA—EDTA are relatively stable; however, penicillamine in urine is rapidly oxidised. In common with others [5] we have found that the addition of a small quantity of EDTA at the time of sample collection prevents the oxidation. A time course experiment showing the disappearance of penicillamine in urine, with and without added EDTA, is shown in Fig. 5.

However, even at the point of collection, urine (and plasma) samples containing penicillamine will also contain penicillamine in the oxidised (disulphide) form, as well as mixed disulphides of penicillamine with cysteine and perhaps other —SH compounds. Any technique to determine total levels of penicillamine therefore requires some method of measuring the penicillamine present in disulphide form. Precolumn reduction of the disulphides electrochemically has



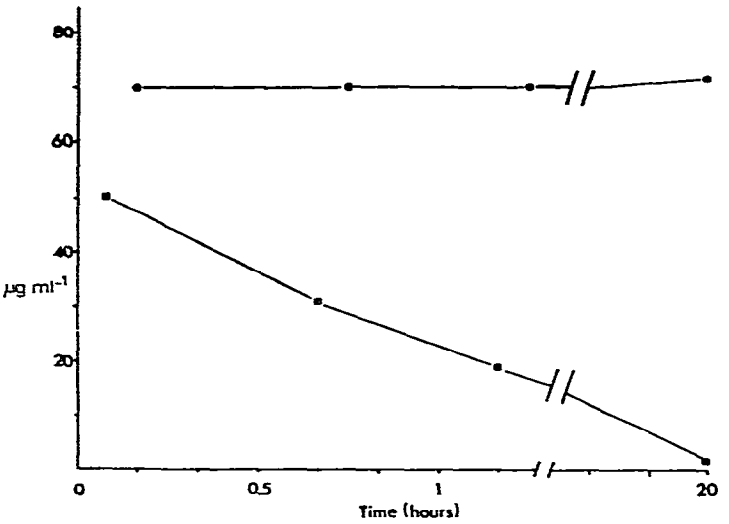


Fig. 4. (Top) Chromatogram of urine from a patient receiving 250 mg of penicillamine per day, 3.5 h after dosing. A = homocysteine, B = penicillamine. Internal standard is homocysteine 5 μg ml^{-1}; absorbance of peak = 0.054. Penicillamine absorbance = 0.103; this corresponds to 11.6 μg ml^{-1} penicillamine. (Bottom) Chromatogram of blank urine.

Fig. 5. Quantity of penicillamine detected in urine in the presence (•) and absence (▪) of EDTA over a period of 20 h.

been reported [5], and the chemical reduction of disulphides using Cleland's reagent (dithioerythritol, DTE) is well established [9]. Our attempts to use DTE to reduce disulphides before chromatographic separation were unsatisfactory because the injection of DTE rapidly destroyed the separating capacity of the analytical columns, and no satisfactory method of removing excess DTE was found. Alternatively, the disulphides might be reduced after chromatography (but before mixing with Ellman's reagent). Indeed post-column reaction and detection of disulphides has been described [10]. The availability of dihydrolipoamide covalently bound to glass beads suggested the possibility of placing a short column of these between the analytical column and the post-column reactor. Preliminary experiments showed that when the disulphides of cysteine and penicillamine were chromatographed on the system free thiols were detected in the eluent. Differences in the reactivity of the two disulphides were found, cystine being much more readily reduced than penicillamine di-

sulphide. Such differences in the reactivity of these two compounds have been noted previously [11], and were attributed to steric factors. Further work to find the best conditions for this disulphide-reducing column is in progress.

CONCLUSION

For the analyst penicillamine poses a number of problems of specific detection and stability. Our experiments with the post-column reaction of thiols with Ellman's reagent provide the basis for a rapid, sensitive and specific method for their analysis in biological fluids. The insensitivity of the system to contaminants means that sample preparation can be kept to a minimum.

Our preliminary experiments with post-column reduction of disulphides are promising and may allow the analysis of both reduced and oxidised forms of the drug in the same sample, without the need for prechromatographic chemistry.

New reagents that show marked changes in UV absorption [12] or fluorescence [13] in the presence of thiols have recently become available. Such compounds substituted for Ellman's reagent may provide even greater sensitivity and specificity. In this context we are currently examing monobromotrimethylammoniobimane and related compounds [13].

In conclusion, the procedures which we have described provide a basis for the analysis of penicillamine (and other thiols) in urine.

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