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Note

Analysis of D-penicillamine by high-performance liquid chromatography with glassy carbon electrochemical detection

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Electrochemical detection in conjunction with high-performance liquid chromatography (HPLC) has mostly been applied to the analysis of catecholamines and related compounds. The use of glassy carbon electrodes in electrochemical detection for the analysis of thiol compounds is a new application of the technique and underlines the importance of electrochemical detection in fields other than those related to catechols.

Penicillamine (α-amino-β-methyl-β-mercaptobutanoic acid) is a compound of importance in the therapy of several diseases, including rheumatoid arthritis [1], Wilson's disease [2] and in the treatment of heavy metal poisoning [3].

Measurement of low concentrations of penicillamine in biological fluids presents certain difficulties, in that amino acids and naturally occurring thiol compounds interfere with most assays, and the highly polar nature of both penicillamine and the interfering substances makes separation difficult. Several HPLC techniques for the analysis of penicillamine have been reported recently, they incorporate various detection techniques, including fluorescence derivatisation [4, 5], post-column reaction [6] with Ellman's reagent, performic acid oxidation followed by amino acid analysis procedures [7] and electrochemical detection utilising a mercury-based electrode [8].

Of these, the optical methods require derivatisation before or after chromatography; some are time consuming. The Ellman post-column technique [6] requires a post-column reactor which utilises an extra HPLC pump, and suffers from limited sensitivity which makes it useful for urine analysis, but not for plasma.

Electrochemical detection employing a mercury-based electrochemical detector has been applied to penicillamine assay [7]; however the mercury-

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based electrode employed is difficult to maintain and prone to sudden sensitivity changes. The glassy carbon electrode on the other hand is simple to maintain and has a long life during which its detection sensitivity changes little. The glassy carbon electrode has the added advantage that when operated at a potential of +800 mV non-thiol endogenous compounds present in plasma samples appear not to interfere with analysis of blood samples.

MATERIALS AND METHODS

Chemicals

D-Penicillamine, L-cysteine, DL-homocysteine and glutathione were purchased from Sigma (Poole, Great Britain). 1-Heptanesulphonic acid sodium salt (HSA) was purchased from Magnus Scientific (Sandbach, Great Britain). All other chemicals were of general laboratory reagent grade.

Collection and preparation of blood samples for HPLC

Blood samples (5 ml) were collected from three patients receiving penicillamine (125 mg daily) for the therapy of rheumatoid arthritis, and from volunteers not receiving the drug. The samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) (G.D. Searle & Co., High Wycombe, Great Britain; blood tubes KE/5). The presence of EDTA was necessary to inhibit the blood clotting process and to prevent formation of penicillamine disulphide on storage. As soon after collection as possible the plasma was separated by centrifugation ($2 \cdot 10^3 g$, 10 min) and the plasma proteins precipitated by the addition of trichloroacetic acid (TCA) (200 μl plasma plus 200 μl 18% w/v TCA), followed by standing at 0°C for 10 min. The precipitated protein was removed by centrifugation, and the clear supernatant analysed by HPLC with electrochemical detection, as described below.

It was necessary to precipitate plasma proteins and maintain acid conditions to permit storage (i.e. for several hours) of the penicillamine-containing solution without considerable losses of penicillamine [9].

HPLC equipment

The HPLC system comprised a Constametric III solvent pump (Laboratory Data Control, Stone, Great Britain), a Rheodyne injection valve (Rheodyne, Berkeley, CA, U.S.A.), a Hypersil ODS 25 cm × 4 mm I.D.; particle size 5 μm analytical column (Magnus Scientific) equilibrated with heptanesulphonic acid [6], detection was electrochemical at a potential of +800 mV. The electrochemical detector (LCA 15) and glassy carbon electrode were purchased from EDT Research (London, Great Britain).

Elution solvent

The mobile phase, previously described by Beales et al. [6], consisted of aqueous phosphate buffer (0.25 M; pH 7.4) containing EDTA ($4 \cdot 10^{-4} M$) and HSA ($3.5 \cdot 10^{-4} M$). It was used at a flow-rate of 1 ml min^{-1}.

Investigation of endogenous thiols and penicillamine

Aliquots (10 μl) of solutions (10 μg ml^{-1} in 100 mg% aqueous EDTA) of

authentic penicillamine, cysteine, homocysteine and glutathione (solutions prepared immediately before use) were injected onto the analytical column in order to determine the retention volumes of these compounds. Samples (10 μl) of diluted and deproteinised plasma were injected for plasma level determinations.

Calibration graph

A calibration graph was prepared for absolute penicillamine levels between 5 and 20 ng injected onto the column.

Assessment of accuracy

Samples (n = 9) containing 10 ng of D-penicillamine in 10 μl of 100 mg% EDTA were analysed by the above technique. Quantitative variation in the analytical procedure was calculated from these data.

RESULTS AND DISCUSSION

Penicillamine was readily detectable at a glassy carbon electrode at a potential of +800 mV (Fig. 1B). The penicillamine eluted with k' = 1.5 (V_0 = 1.6 ml), gave a linear calibration between 5 and 20 ng in a 10-μl sample, above this value the graph formed a plateau. The detection limit for the technique was 2 ng (i.e. 0.4 μg cm^{-3} plasma) in comparison with 10 ng for the Ellman post-column colorimetric detection system [6].

Comparison of deproteinised plasma samples from volunteers not given penicillamine with patients receiving penicillamine for the treatment of rheumatoid arthritis, demonstrated a peak with a k' corresponding to penicillamine in the latter group (Fig. 1A). Interfering compounds which co-chromatograph with penicillamine were not present in the control plasma. Plasma levels of free penicillamine in three patients receiving a daily dose of 125 mg of penicillamine were 2.5 ± 0.5 μg ml^{-1} (mean ± S.D.), approximately 8—9 h after the penicillamine dose.

Interference by naturally occurring thiols was found not to be a problem since cysteine (k' = 0), homocysteine (k' = 0.3) and glutathione (k' = 0.1) all eluted at a very short retention volume, and thus were well separated, chromatographically, from penicillamine (k' = 1.5).

Since electrochemical detection of penicillamine relies upon oxidation of the thiol group, its known metabolites, namely penicillamine disulphide and S-methylpenicillamine [10] were not detectable, this technique is therefore specific for penicillamine per se.

Quantitative variation in the analytical procedure was determined for nine samples of 10 ng penicillamine in 10 μl total injection volume. The meter deflection for these samples was 10.8 ± 0.73 nA, therefore the intra-sample variation was 13.5%. Penicillamine is readily oxidised in aqueous solution to form penicillamine disulphide. This reaction is inhibited by acid conditions in the presence of EDTA [9]; however, in order to minimise inaccuracies in quantification it is essential to prepare standard solutions of, and a standard curve for, penicillamine daily and to analyse plasma samples within 1 h of their collection and deproteination.

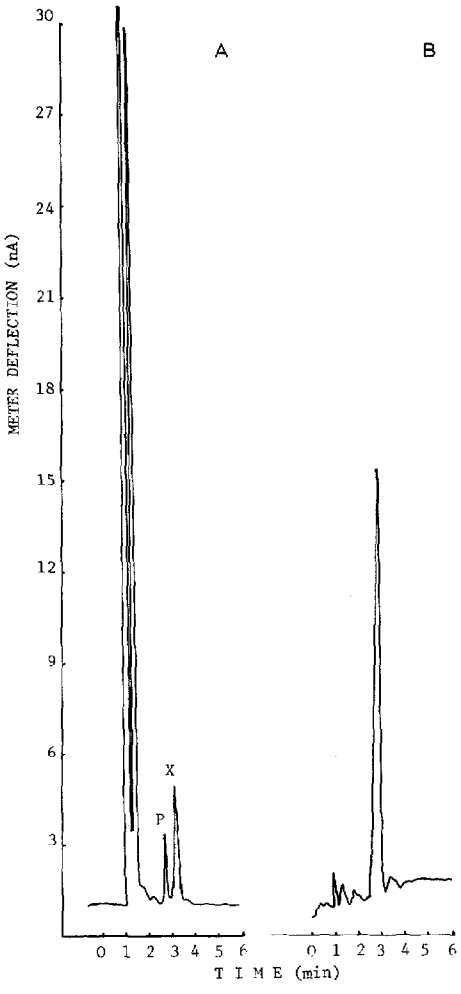

Fig. 1. (A) Analysis of a plasma sample from a patient receiving D-penicillamine using a reversed-phase HPLC column eluted with phosphate buffer (0.25 *M*; pH 7.4) containing EDTA and HSA (flow-rate = 1 ml min^{-1}) followed by electrochemical detection. Peaks: P = penicillamine; X = unidentified endogenous compound also present in control plasma samples. (B) Analysis of 15 ng of authentic D-penicillamine by the same technique as described above.

While ion pairing with HSA seems improbable as the mechanism of penicillamine retention at the pH used in the analytical procedure, good resolution and peak shape for penicillamine in biological samples were not found unless HSA was present. It seems possible that the HSA—octadecyl combination effectively resembles an ion-exchange column and that penicillamine retention was by an ion-exchange mechanism.

HPLC with glassy carbon electrochemical detection is therefore a technique which may be applied to the analysis of penicillamine. The procedure is of high sensitivity and thus may be used for the analysis of plasma samples obtained from patients receiving penicillamine for the treatment of rheumatoid

arthritis. The technique therefore has applications in the routine clinical laboratory and in studies on pharmacokinetics in the research laboratory.

The observations that cysteine, homocysteine and glutathione are electrochemically active underlines the potential usefulness of electrochemical detection at a glassy carbon electrode for the detection of thiol compounds, generally, an application which has received little attention to date.

CONCLUSIONS

A method for the determination of penicillamine in plasma is described. The method utilises reversed-phase HPLC with electrochemical detection at a glassy carbon electrode (+800 mV). The HPLC system used comprised a C_{18} reversed-phase column (Hypersil ODS) and used phosphate buffer (pH 7.4) containing heptanesulphonic acid ($3.5 \cdot 10^{-4} M$) and EDTA ($4 \cdot 10^{-4} M$) as the mobile phase. A high degree of linearity was found within the on-column injection range 5—20 ng. For 10-ng samples the electrochemical detection response was 10.8 ± 0.73 nA ($n = 9$).

The technique provides a rapid, sensitive method for determining penicillamine in the plasma of patients being treated with the drug.

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