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MEASUREMENT OF PENICILLAMINE AND N-ACETYLCYSTEINE IN HUMAN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid and precise high-performance liquid chromatographic assay for both N-acetylcysteine and penicillamine in blood samples is described using selective reductive electrochemical detection and a high-efficiency C_{18} reversed-phase column. The use of an internal standard compensated for changes in detector responses during a run and for variable sample recovery. The detection limits for N-acetylcysteine and penicillamine were 25 and 10 ng/ml, respectively, using 500-μl blood samples. Reproducibility of measurement for both thiols was excellent. This method allows routine monitoring of blood levels and pharmacokinetic studies with N-acetylcysteine and penicillamine.

INTRODUCTION

The thiols penicillamine and N-acetylcysteine are of considerable therapeutic importance. For example, penicillamine is widely used for the treatment of Wilson's disease, cystinuria, and rheumatoid arthritis [1, 2] whilst N-acetylcysteine is used as a mucolytic drug in the treatment of bronchitis [3] for the emergency treatment of paracetamol overdosage [4] and more recently to potentiate the action of nitroglycerin therapy [5]. Both drugs have also been used to treat a variety of heavy metal toxicities [2, 6] and are potentially toxic. It is, however, extremely unusual for the two drugs to be used in the same patient which allows each to be used as an internal standard in the assay of the other.

There is a need to determine the bioavailability and pharmacokinetics of these thiols and to monitor levels during chronic therapy. Published analytical

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methods specific for these thiols, however, have not been totally satisfactory. Methods for the measurement of penicillamine have generally used gas- liquid chromatographic [7, 8] or high-performance liquid chromatographic (HPLC) [9—11] techniques in which the drug is first derivatized prior to chromatography. Methods for N-acetylcysteine are uncommon and are generally long due to chemical derivatization procedures [12, 13].

More recently direct electrochemical detection of the sulfhydryl of penicillamine [14—16] has become the method of choice due to its high selectivity for thiols and the ease of operation of the detector and additionally because little sample preparation is required. Published electrochemical techniques for penicillamine have so far used ion-exchange columns for the separation process. These have a relative low efficiency and thiols such as N-acetylcysteine are eluted in the solvent front. The lack of an internal standard in published methods has also limited the precision of the determination due to variable detector responses during chromatography and variable recoveries from one sample to the next.

We report here a quick and reliable reductive electrochemical HPLC method for the measurement of penicillamine and N-acetylcysteine using the other analyte as internal standard in blood and employing a high-efficiency reversed-phase C_{18} bonded column. Blood levels of both thiols are shown from patients receiving these drugs. The method is also suitable for the simultaneous determination of reduced glutathione in blood samples.

EXPERIMENTAL

Reagents

Penicillamine (gift from Eli Lilly, Australia), and N-acetylcysteine (Sigma, St. Louis, MO, U.S.A.) were prepared daily as 1 mg/ml stock solutions in 1 mg/ml EDTA in distilled water. Dilutions were made as required in 1 mg/ml EDTA. All other reagents were analytical-reagent grade.

Chromatographic system

A liquid chromatograph fitted with a Waters Model M45 solvent delivery system, a Rheodyne loop injector and a Bioanalytical Systems LC-4 electrochemical detector equipped with a gold/mercury electrochemical cell were used. The gold/mercury cell was maintained at +0.17 V versus an Ag/AgCl reference electrode. The mobile phase consisted of 4% methanol in 0.1% (v/v) phosphoric acid and 10 m*M* sodium sulfate which was refluxed under nitrogen for at least 4 h prior to use. The mobile phase was continually purged with nitrogen during use. All plastic solvent lines were replaced with stainless-steel tubing to prevent diffusion of oxygen into the mobile phase. The background current was typically 10 nA and the sensitivity of the detector was usually set at 500 mV.

The column consisted of a NOVA column (15 cm × 3.9 mm I.D.) packed with a 5 μm particle size μBondapak C_{18} phase (Waters Assoc.). No guard column was used for these experiments. The mobile phase was pumped at 0.5 ml/min and was recycled for upto three days before it was replaced.

Sample preparation

Freshly drawn blood collected in EDTA-coated tubes was added to ice-cold disposable glass tubes containing 200 μl of an EDTA solution (1 mg/ml), 250 μl of perchloric acid (1.0 *M*) and internal standard. After brief mixing the solution was kept on ice and centrifuged at 1000 *g* maximal for 5 min. The supernatant (500 μl) was then added to a further ice-cold disposable glass tube containing 75 μl of a pH 9.0 potassium phosphate solution (0.8 *M* in 1 mg/ml EDTA solution). The pH of the sample should be about 2.5. After centrifugation for 5 min at 1000 *g* maximal an aliquot of the supernatant (5- 25 μl) was injected into the liquid chromatograph.

Plasma samples

Drug-free venous blood which was obtained from healthy subjects was used for construction of calibration curves. Blood was obtained both from five outpatients receiving chronic penicillamine therapy (250 mg twice daily) for the treatment of rheumatoid arthritis and from patients with angina pectoris participating in a clinical study in which interactions between N-acetylcysteine and nitroglycerine therapy were being investigated.

Pharmacokinetic analysis

Plasma concentration–time data were fitted to a polyexponential equation using a non-linear least-squares regression analysis computer program, AUTOAN2 [17], assuming first-order absorption. Equations generated by this program included a lag time for oral absorption.

RESULTS AND DISCUSSION

The overall recovery was evaluated by spiking blood with known amounts of penicillamine and N-acetylcysteine. The recovery was then evaluated by calculating the recovered penicillamine and N-acetylcysteine and comparing these with known standard injections of the two drugs. The recoveries of penicillamine and N-acetylcysteine in blood were 66 ± 6% and 49 ± 5% (mean ± S.D., $n = 4$), respectively.

The addition of an aliquot (usually 500 μl) of freshly collected blood to tubes already containing internal standard, EDTA solution and perchloric acid

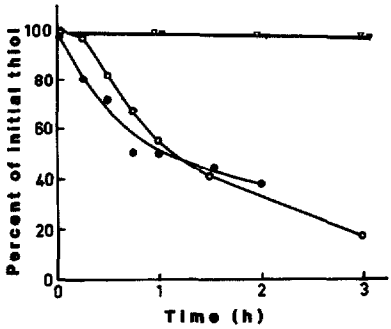

Fig. 1. Time course showing the stability of 10 μg/ml penicillamine (•) and N-acetylcysteine (○) in human blood incubated at 4°C and the stability of penicillamine (▾) and N-acetylcysteine (▽) after perchloric acid acidification.

minimized loss of thiols by oxidation. If blood was not treated in this way blood concentrations of both penicillamine and N-acetylcysteine declined rapidly with a half-life of about 60 min at 4°C (Fig. 1). However, once treated with acid and EDTA both thiols were stable for at least 3 h (Fig. 1) which is consistent with the observations of Saetre and Rabenstein [15]. This permitted collection of several blood samples before chromatography was necessary. The final perchloric acid extract was stable when kept on ice and could be stored overnight at 4°C without significant loss of thiols.

The addition of internal standard largely compensated for any slight loss of thiols during sample preparation and storage and provided a real advantage over previously reported analytical methods which have only attempted to measure either penicillamine or N-acetylcysteine individually without the use of an internal standard [12, 15].

The mobile phase (4% methanol in 0.1% phosphoric acid and 10 m*M* sodium sulfate) and type of column (5 μm particle size C_{18}) have been optimized to allow chromatography of both penicillamine and N-acetylcysteine in one run. The retention times for these thiols were 5.0 and 7.3 min, respectively (Fig. 2). Column efficiences were significantly higher than previously reported methods using ion-exchange columns [14, 15]. Both thiols give almost symmetrical peak shapes under these conditions whereas previous methods using ion-exchange resins were not able to sufficiently resolve N-acetylcysteine from the solvent front. In this procedure N-acetylcysteine elutes last (after penicillamine) and well after many endogenous thiols such as cysteine and glutathione. No interfering peak has been observed in the eluate regions of penicillamine and

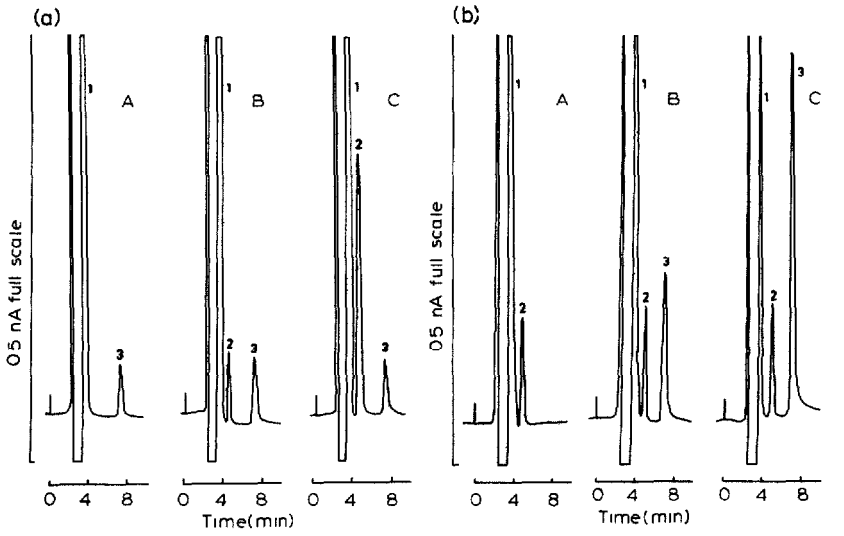

Fig. 2. (a) Chromatograms of penicillamine using N-acetylcysteine (2 μg) as internal standard: (A) drug-free blood extract to which internal standard has been added; (B) blood extract from a patient receiving penicillamine, equivalent to 1.70 μg/ml; (C) 7.5 μg/ml penicillamine standard. (b) Chromatograms of N-acetylcysteine using penicillamine as internal standard: (A) drug-free blood extract to which internal standard has been added; (B) 8.0 μg/ml N-acetylcysteine standard; (C) blood extract from a patient receiving N-acetylcysteine, equivalent to 19.8 μg/ml. Peaks: 1 = glutathione; 2 = penicillamine; 3 = N-acetylcysteine; injection event marker shown at zero time.

N-acetylcysteine. Thiols tested for possible interference include glutathione, cysteine, cysteamine, 2-mercaptoethanol, thiomalic acid, 2-mercaptoacetic acid and 2-mercaptopropionic acid. In addition there was no evidence of absorption of thiols on the column or at the injection port which could lead to carry-over of thiols from subsequent injections as reported by Perrett and Rudge [18]. The improvement probably relates to the use of a reversed-phase column in which all reactive sites have been inactivated by a capping method.

Calibration curves were constructed by adding thiol to drug-free blood. For the measurement of penicillamine, N-acetylcysteine (2 μg per tube) was used as an internal standard. Conversely when measurement of N-acetylcysteine blood levels was required penicillamine was used as the internal standard. A typical standard curve for penicillamine relating the peak-height ratio of penicillamine to N-acetylcysteine (y) and penicillamine blood levels in μg/ml (x) was expressed by the equation $y = 1.513x - 0.0374$ ($r^2 = 0.9964$). The calibration curve was linear up to at least 10 μg/ml. A typical calibration curve expressing peak-height ratios of N-acetylcysteine to penicillamine (y) against blood levels of N-acetylcysteine in μg/ml were $y = 0.1884x - 0.0411$ for N-acetylcysteine standards in the range 0—10 μg/ml using 2 μg penicillamine as internal standard. When higher blood levels of N-acetylcysteine are encountered such as after a 100 mg/kg infusion, the amount of internal standard was increased to 40 μg per tube. Calibration curves were linear up to at least 160 μg/ml. A typical calibration curve in the range 10—160 μg/ml was $y = 0.011x - 0.054$ ($r^2 = 0.985$).

The precision of each method was determined by replicate analysis of known amounts of penicillamine or N-acetylcysteine added to drug-free blood. For penicillamine, the coefficients of variation for replicate analyses of 500 ng/ml and 1 μg/ml blood samples were 7.8% ($n = 10$) and 5.0% ($n = 12$), respectively. Similarly, for N-acetylcysteine the coefficients of variation for replicate analyses of 2 and 10 μg/ml blood samples were 9.6% ($n = 11$) and 3.3% ($n = 13$), respectively.

The detection limits (twice background noise) of penicillamine and N-acetylcysteine were 10 and 25 ng/ml, respectively, using 50-μl injection volumes and the optimum voltage of +0.17 V with respect to the working electrode. These detection limits correspond to 0.19 and 0.45 nmol for penicillamine and N-acetylcysteine, respectively.

Glutathione was also detected in all blood samples and eluted at 4 min in the chromatogram (Fig. 2). Due to the large endogenous concentration of glutathione the initial sensitivities used for detection of penicillamine and N-acetylcysteine gave off-scale peaks for glutathione. It was possible, however, to reduce either the injection volume or sensitivity on repeat injections and then quantitate for blood glutathione levels. If required, the proportion of methanol in the mobile phase can be reduced to further resolve the glutathione peak from the solvent front. However, this did not greatly change the results obtained using the original mobile phase conditions.

Blood levels

Blood levels of penicillamine were measured in five patients who had been on chronic penicillamine therapy for rheumatoid arthritis. A single 250-mg oral

dose of penicillamine was given on the morning of the study. Pharmacokinetic analysis of the data is consistent with a one-compartment open model with first-order absorption. The computer fitted curve (Fig. 3) shows mean peak levels of 1.04 μg/ml (S.E. = 0.07) at 1.83 h (S.E. = 0.11) and an elimination half-life of 2.09 h (S.E. = 0.20). At 8 h the mean blood penicillamine level was still detectable at 0.18 μg/ml (S.E. = 0.06). These results are consistent with previous reports which examined the pharmacokinetics of D-penicillamine in patients undergoing chronic therapy [19]. The double-peak effect previously seen with penicillamine [20] was also seen in three of the five subjects. How-

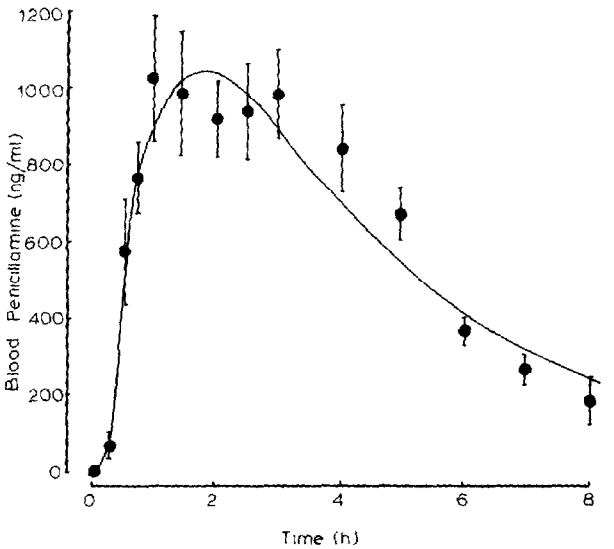

Fig. 3. Mean blood levels of penicillamine in five patients with rheumatoid arthritis on chronic penicillamine therapy given a 250-mg oral dose of penicillamine. Bars are S.E.M.

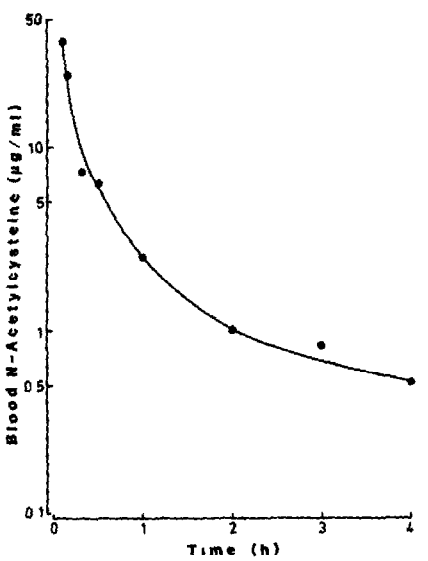

Fig. 4. Blood levels of N-acetylcysteine in one patient after receiving a 1-g bolus intravenous dose of N-acetylcysteine.

ever, the appearance of the second peak was not considered significant, hence a smooth curve was fittted to the data by computer analysis.

A bolus intravenous dose (1 g) of N-acetylcysteine administered to a volunteer suffering from stable angina pectoris gave rise to readily detectable blood levels of N-acetylcysteine. At 5 min the level was 37 μg/ml which rapidly declined to 0.5 μg/ml at 4 h post-dose (Fig. 4). Pharmacokinetic analysis suggested a two-compartment open model with a terminal elimination half-life of 1.36 h.

In summary, a rapid and precise HPLC assay for both N-acetylcysteine and penicillamine in blood samples is described using reductive electrochemical detection. N-Acetylcysteine is used as internal standard to compensate for changes in detector response and sample recovery for analysis of penicillamine blood levels and when N-acetylcysteine blood levels are required penicillamine can be used as internal standard. The method has been found to be sufficiently sensitive and reliable to study the pharmacokinetics of N-acetylcysteine and penicillamine in blood samples taken from patients participating in clinical studies on the drugs.

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