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ANALYSIS OF D-PENICILLAMINE IN PLASMA BY FLUORESCENCE DERIVATISATION WITH N-[*p*-(2-BENZOXAZOLYL)-PHENYL] MALEIMIDE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic assay for penicillamine in plasma is described. The method is based on the derivatisation of penicillamine in acidified protein-free plasma supernatants with the sulphydryl-specific reagent N-[*p*-(2-benzoxazolyl)-phenyl] maleimide (BOPM) to give a stable fluorescent product. After separation of the penicillamine—BOPM derivative by reversed-phase high-performance liquid chromatography, fluorescence detection enables the quantitation of plasma penicillamine concentrations in the range 0.25—500 μmol/l. The method is selective and reproducible, and since chromatography time is less than 7 min the method is readily applicable to the analysis of the large number of samples associated with pharmacokinetic studies.

INTRODUCTION

Although D-penicillamine (β,β-dimethylcysteine) was originally introduced for the treatment of Wilson's disease, a rare disorder of copper metabolism, more recently it has found widespread acceptance for the treatment of rheumatoid arthritis. Despite the favourable effects of penicillamine in rheumatoid disease, use of the drug is associated with a high incidence of side effects. However, the relationship between plasma penicillamine concentration,

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clinical efficacy and the occurrence of adverse effects has still to be investigated.

To date, detailed studies on the pharmacokinetics of penicillamine in man have been impeded by the lack of suitable analytical methodology. Methods developed for the determination of penicillamine include radioimmunoassay [1], colourimetry [2, 3] and gas—liquid chromatography [4]. Separation and detection based on an automated amino acid analyser have also been described [5]. These procedures are all generally unsatisfactory for pharmacokinetic studies, however, since they are either non-selective, lack the required sensitivity or require complex sample manipulation. In recent years the convenience and versatility of high-performance liquid chromatography (HPLC) has led to its acceptance as one of the most useful techniques available for the analysis of drugs in biological fluids [6]. Recently assays for penicillamine using HPLC with electrochemical detection have been described [7, 8] but problems inherent in the use of this type of detector have precluded the widespread acceptance of such procedures. Similarly, a liquid chromatographic method based on post-column derivatisation of penicillamine with Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] requires a specially constructed reaction cell [9].

Fluorescence derivatisation of the sulphydryl group of penicillamine provides a means of both preventing oxidation of the compound during analysis and enhancing assay sensitivity. An HPLC method for penicillamine based on formation of the fluorescent dansylaziridine derivative has been reported [10] but this procedure has been difficult to reproduce (see Results and discussion). Thus, we have developed a simple procedure for the analysis of penicillamine in plasma based on reaction of the sulphydryl group with N-[*p*-(2-benzoxazolyl)-phenyl] maleimide (BOPM) to give a stable fluorescent derivative which may be separated by reversed-phase HPLC. The assay is simple, specific, sensitive and reproducible, and readily applicable to pharmacokinetic studies.

EXPERIMENTAL

Reagents and standards

D-Penicillamine was purchased from Sigma (St. Louis, MO, U.S.A.) and N-[*p*-(2-benzoxazolyl)-phenyl] maleimide from Eastman-Kodak (Rochester, NY, U.S.A.). Other reagents and solvents were of analytical grade.

A penicillamine stock solution was prepared each day by dissolving the pure standard in 0.1% ethylenediamine tetraacetic acid (EDTA) to give a final concentration of 5 mmol/l. The stock solution was further diluted with 0.1% EDTA to give solutions containing 1000, 500, 250, 100, 50 and 10 μmol/l of penicillamine. Penicillamine calibration standards were then prepared by making a 10-fold dilution of the aqueous standard solutions with drug-free plasma to give final concentrations of 100, 50, 25, 10, 5 and 1 μmol/l, respectively. Upon preparation the penicillamine plasma standards were immediately treated according to the procedure described below under *Sample preparation.*

Chromatography

The HPLC system used consisted of a Model U6K injector, a Model 6000A solvent delivery system (both Waters Assoc., Milford, MA, U.S.A.) and a Model 970 fluorescence detector (Spectra-Physics, Santa Clara, CA, U.S.A.). The excitation wavelength of the fluorescence detector was set at 319 nm and emission was measured using a 360-nm cut-off filter. The chromatograph was fitted with a 30 cm × 3.9 mm I.D. 10-μm reversed-phase μBondapak C_{18} column (Waters Assoc.) and operated at ambient temperature. The mobile phase was methanol—sodium acetate, 0.1 m*M* (48:52) used at a flow-rate of 2.0 ml/min.

Sample preparation

After collection, patient blood samples were rapidly transferred into 1.5-ml Eppendorf microtubes and centrifuged at 6500 *g* for 30 sec (Eppendorf Model 5412 high-speed centrifuge) to separate the plasma. A 1-ml aliquot of plasma, from the patient samples or standards, was then immediately transferred into a second Eppendorf microtube containing 0.15 ml of 25% trichloroacetic acid. The acidified plasma was vortex mixed, cooled on ice for 10 min (to complete the plasma protein precipitation process) and the protein separated by centrifugation at 6500 *g* for 2 min. The penicillamine in plasma treated in this manner and stored at −20°C is stable for at least ten days.

For the derivatisation process, 0.5 ml of the plasma supernatant in a 5-ml glass culture tube was neutralised with 0.2 ml of 1% aqueous sodium hydroxide solution and the pH of the solution was adjusted to 5.0 by the addition of 0.25 ml of 0.5 mol/l sodium citrate (titrated to pH 5.0 with perchloric acid). To this buffered solution was added 1.0 ml of the derivatising agent (BOPM, 1 mmol/l in ethanol) and the mixture was incubated at 37°C overnight. A 0.05-ml aliquot of the reaction mixture was injected directly into the chromatograph.

Unknown concentrations were determined by comparison of the penicillamine peak heights obtained from the patient samples with those of the calibration curve.

RESULTS AND DISCUSSION

Sample preparation

Using the HPLC procedure reported here, we confirmed the previous results of Bergstrom et al. [11] that plasma samples must be acidified and deproteinised immediately upon collection to avoid the loss of reduced penicillamine. Thus, for an untreated 50 μmol/l plasma standard stored at room temperature the half-life for the rate of penicillamine loss, presumably by oxidation to the disulphide, was found to be only 15 min. However, when plasma samples were treated according to the procedure outlined in the Experimental section, minimal (< 5%) loss of penicillamine occurred when the acidified, protein-free supernatants were stored frozen for ten days. Although EDTA was used to prevent oxidation of penicillamine in the aqueous standard stock solutions, it was demonstrated that the presence of EDTA did not significantly improve the stability of penicillamine in the acidified plasma samples. Indeed, reaction mixture EDTA concentrations in excess of 5 mmol/l

adversely affected the chromatography of the penicillamine-BOPM derivative.

It was also shown by HPLC that minimal penicillamine loss occurred from plasma samples during the sample preparation procedure. Thus, the mean (± S.D.) peak heights obtained from quadruplicate plasma standards containing 50 and 5 μmol/l of penicillamine compared to stabilised aqueous standards of the same concentrations were 96.6 ± 2.3% and 96.0 ± 3.1%, respectively.

Derivatisation

Since penicillamine does not significantly absorb energy in the ultraviolet or visible region of the spectrum, the detection of the drug at the concentrations normally found in plasma requires derivatisation of one of the functional groups of penicillamine with a suitable chromophoric reagent or else utilisation of other physico-chemical properties of the molecule (e.g. electrochemical oxidation). The approach followed in developing the present assay was derivatisation of the sulphydryl group of penicillamine with a chromophoric reagent, thereby providing a means of both detecting the compound and preventing autoxidation during chromatography. Numerous reagents previously reported as being suitable for the derivatisation of sulphydryl-containing amino acids were investigated. These included: *o*-phthalaldehyde, dinitrolfluorobenzene, Dns-aziridine and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride). However, derivatisation of sulphydryl groups with each of these reagents requires a weakly alkaline reaction medium (pH 8—9), conditions known to catalyse penicillamine disulphide formation. Only the reaction of *o*-phthalaldehyde with penicillamine proceeded rapidly enough to minimise the problems of poor sensitivity and reproducibility arising from competing penicillamine autoxidation. Even then, due to the non-specificity of *o*-phthalaldehyde as a derivatising agent, the penicillamine—phthalaldehyde complex could not be adequately resolved from the large number of endogenous plasma peaks using reversed-phase HPLC.

The importance of pH control during the derivatisation process was confirmed in an experiment which determined the rate of penicillamine loss with increasing alkalinity. Here aliquots of a 500 μmol/l penicillamine aqueous standard solution (in 0.1% EDTA, see *Reagents and standards* section) were buffered to either pH 5.0, 7.0, 8.0 or 9.0 and the reduced sulphydryl concentration determined by Ellman's procedure [12] at known intervals after buffer addition. The results of this experiment are summarised in Fig. 1 and clearly demonstrate the instability of penicillamine in neutral and alkaline solutions at room temperature.

The reaction of maleimides with sulphydryl groups is known to proceed under mildly acidic conditions [13, 14] and the use of compounds of this class offers obvious advantages for the derivatisation of the penicillamine sulphydryl group. In particular, BOPM was investigated as a potential penicillamine derivatising agent since the compound is an established labelling reagent for sulphydryl-containing proteins [15, 16]. The reaction of penicillamine with BOPM at pH 5.0 readily formed a fluorescent product, with absorbance and emission maxima at 319 and 368 nm, respectively. At least a 4- to 5-fold excess of BOPM was shown to be necessary for the derivatisation

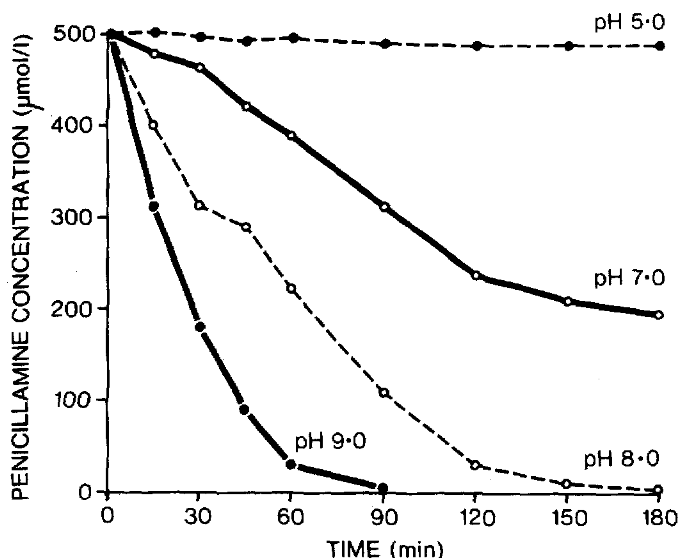

Fig. 1. Rate of loss of penicillamine from a 500 µmol/l aqueous standard solution buffered to pH 5.0, 7.0, 8.0 or 9.0.

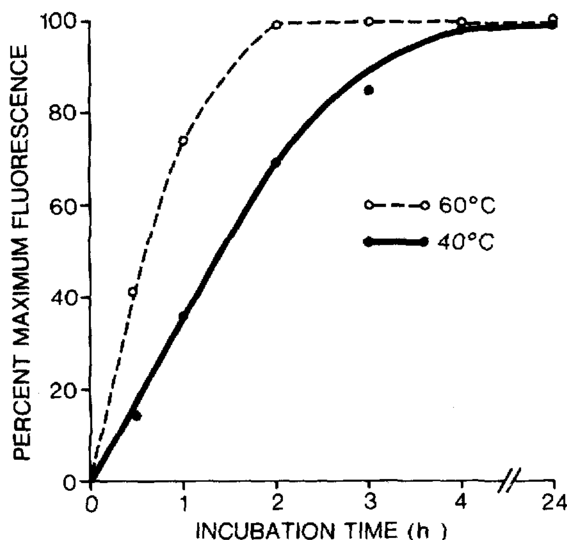

Fig. 2. Time course of the reaction of BOPM with a 50 µmol/l penicillamine plasma standard at 40° C and 60° C. Sample preparation and chromatography conditions as outlined in the Experimental section.

of penicillamine in deproteinised plasma samples. The time course of the extent of reaction of BOPM with a 50 µmol/l penicillamine plasma standard at 40°C and 60° C was followed by HPLC and indicated that the rate of formation of the penicillamine–BOPM complex was temperature dependent (Fig. 2). Although the reaction was complete within 2 h at 60°C or 4 h at 40°C, the normal procedure adopted in this laboratory has been to carry out the derivatisation overnight in a 37° C incubator. Once formed, the penicillamine–

BOPM complex is stable for 24 h (Fig. 2) and if stored in the dark less than 5% decomposition occurs over 60 h.

Since BOPM is a sulphydryl-specific reagent neither penicillamine disulphide nor penicillamine—cysteine disulphide, the major metabolites of penicillamine, interfere with the assay procedure. The extent of disulphide formation may, however, be determined by the method described here following electrochemical reduction of the disulphides [7] and measurement of total penicillamine. No endogenous plasma constituents interfere with the quantitation of penicillamine and the BOPM complexes formed by cysteine and glutathione have been shown to be unretained under the chromatography conditions employed. When the derivatisation is performed under alkaline conditions at least three fluorescent products were shown to be formed. The formation of one of these products is penicillamine-independent and may represent hydrolysis of BOPM to its maleamic acid derivative.

Chromatography

The penicillamine—BOPM derivative chromatographs well on reversed-phase HPLC with methanol—0.1 mmol/l sodium acetate as the mobile phase. The complex elutes as a sharp, symmetrical peak with a retention time of 5.5 min.

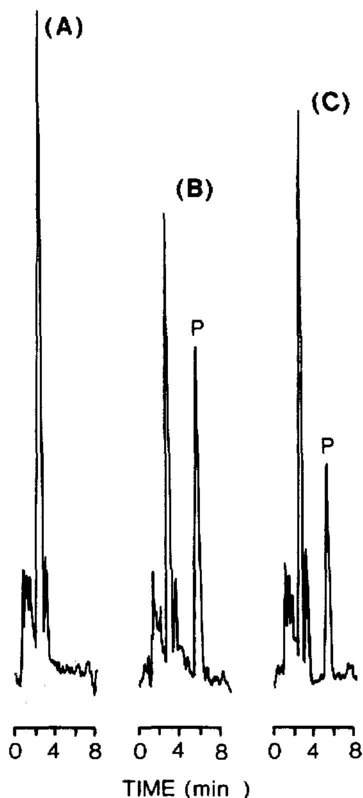

Fig. 3. Chromatograms of plasma samples. (A) Drug-free plasma; (B) plasma standard containing 10 μmol/l of penicillamine (P); (C) patient plasma sample containing 6.5 μmol/l of penicillamine. Detector sensitivity 0.05 μA. Sample preparation and chromatography conditions as outlined in the Experimental section.

Fig. 3B shows the chromatogram of a plasma standard containing 10.0 μmol/l of penicillamine. Similarly, Fig. 3C shows the chromatogram of a plasma sample, taken 6 h after a dose from a patient on chronic penicillamine therapy (250 mg, 12 hourly), containing 6.5 μmol/l of penicillamine. Drug-free plasma gave no interfering peaks under the chromatography conditions described (Fig. 3A).

The penicillamine—BOPM derivative exhibits a marked capacity for ion-pairing with carboxyl anions. Any masking of the charged amino function of the penicillamine—BOPM derivative by carboxyl ions leads to a profound reduction in the polarity of the complex. Low concentrations of sodium acetate are therefore routinely added to the mobile phase to avoid changes in chromatography during the course of analysis.

Unreacted BOPM is sufficiently lipophilic to be retained on the reversed-phase column with the mobile phase used for the assay. Retention of this compound is advantageous since it allows successive injections to be made without interference from later eluting peaks. The accumulation of BOPM on the column does not have any major adverse effects, although baseline drift may increase after approximately 80 injections. Thus, methanol should periodically be pumped through the column to elute retained BOPM.

Assay sensitivity and reproducibility

Optimal sensitivity was obtained when the excitation wavelength of the fluorescence detector is set at 319 nm and a 360 nm cut-off filter used. Under these conditions the limit of sensitivity (signal-to-noise ratio 5:1) for the detection of penicillamine is 0.25 μmol/l. The standard curve is linear for penicillamine plasma concentrations over the range 1—500 μmol/l and passes through the origin.

Although no internal standard is used in this procedure, assay reproducibility is nevertheless good. The mean (± S.D.) coefficient of variation for normalised peak heights (i.e. peak height/concentration) from 20 standard curves prepared over a period of three months was 6.3 ± 2.1%. While a number of sulphydryl-containing compounds were initially investigated as possible internal standards, the assay does not require extraction or quantitative transfer procedures and it soon became apparent that reproducibility was not compromised by the omission of an internal standard.

In summary, the HPLC method described here for the determination of penicillamine in plasma is sufficiently simple, rapid, sensitive and reproducible to readily enable the analysis of the large number of samples involved in pharmacokinetic studies. The method is currently being applied to a study investigating the relationship between plasma penicillamine concentration and the variability observed in therapeutic response and the occurrence of serious adverse effects.

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