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RAPID AUTOMATED DETERMINATION OF D-PENICILLAMINE IN PLASMA AND URINE BY ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION USING A GOLD ELECTRODE

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SUMMARY

A high-performance liquid chromatographic method for the determination of D-penicillamine in plasma and urine has been developed, based on separation on a cation-exchange resin followed by an amperometric detection of the SH group of D-penicillamine oxidized on a gold electrode. The method has been automated and separations from plasma and urine take 7 and 9 min, respectively. The detection limits are 0.05 μg/ml in plasma and 0.2 μg/ml in urine, with a coefficient of variation of 2.9% ($n = 10$).

INTRODUCTION

D-Penicillamine (D-2-amino-3-mercapto-3-methylbutanic acid) is administered for the treatment of Morbus Wilson, metal intoxications, cystinuria and rheumatoid arthritis, its therapeutic effect being due to the SH group, for example via the formation of metal chelates, the participation in redox reactions or SH and SS exchange. Further possible mechanisms of reactions have been discussed[1]. Because of the high toxicity of L-penicillamine[2,3] only the D-isomer is used for therapeutic administration.

For the specific determination of D-penicillamine in plasma and urine photometric methods with SH-specific reagents[4,5] cannot be used, because other metabolites, such as cysteine and glutathione, also contain SH groups.

Only chromatographic methods are suitable for this determination, and in the absence of a chromophore in the molecule low concentrations cannot be detected in the UV region. Methods based on amino acid analysers[6–9] and subsequent derivatization, for example with ninhydrin, taking at least 100 min per sample, are not suitable for routine application. The thin-layer chromatographic method of Pongor[10] allows a quicker measurement, but it cannot distinguish between D-penicillamine and D-penicillamine disulphide and requires a lengthy sample preparation.

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A sensitive and specific method has been published by Saetre and co-workers[11,12] based on separation on a cation-exchange resin (Zipax SCX, 30 μm) with a pre-column, and detection with a mercury electrode, installed in a self-made flow-through cell. Eggli and Asper[13] described the determination of cysteine and related compounds with a similar method. Both methods[11,13] involve reduction of D-penicillamine disulphide to D-penicillamine to obtain information about the concentrations of these metabolites, when it could be demonstrated that these substances (and also mixed disulphides, such as cysteine-D-penicillamine disulphide) can be present in amounts 3–100-fold that of D-penicillamine in urine or plasma.

SH compounds can be oxidized on a mercury or on a gold electrode[14]. This allows the use of an amperometric detector, which is frequently applied in high-performance liquid chromatography (HPLC) for the selective detection of easily oxidizable compounds (phenols, indoles, aromatic amines, etc.)[15]. Solid-state electrodes (for example, glassy carbon) in these detectors permit optimal cell construction, a wide anodic range (up to 1200 mV), a small basic current and low noise[16].

EXPERIMENTAL

Chemicals

The following substances were used: D-penicillamine (Biochemie, Kundl, Austria), D-penicillamine disulphide (Fluka, Buchs, Switzerland), L-cysteine (Fluka), L-glutathione, reduced (Fluka), titriplex III (Merck, Darmstadt, G.F.R.), diammonium hydrogen citrate (Fluka), metaphosphoric acid (Merck) and phosphoric acid (Merck).

Sample preparation

D-Penicillamine in solution is unstable at pH above 6 and is oxidized by trace amounts of iron(III), especially in the neutral region. Therefore, iron(III) must be masked and a low pH is necessary.

Plasma. To 1 ml of blood are added 100 μl of 10% Titriplex III solution. The mixture is shaken, centrifuged and 1 volume of the supernatant is mixed with 2 volumes of a solution prepared by dissolving 4.39 g of diammonium hydrogen citrate in *ca.* 900 ml of water, adding 100 g of metaphosphoric acid and to 1 l with water. The precipitated protein is centrifuged and the supernatant is analysed. If stored for later analysis, the samples are frozen in liquid nitrogen.

Urine. To 1 ml of urine are added 100 μl of 10% Titriplex III solution and 1 volume of this sample is mixed with 2 volumes of the solution described above for plasma. If turbidity the solution is treated as for plasma. If necessary, the above-mentioned disulphides can be reduced at a mercury cathode.

Standard solutions

Standard solutions are prepared so as to contain 1.0 and 5.0 μg of D-penicillamine plus 100 μg of Titriplex III per millilitre of eluent for plasma and urine, respectively.

HPLC method

The eluent (0.02 *M* diammonium hydrogen citrate solution for plasma or a

0.01 *M* solution for urine, the pH being decreased to 2.2 by means of phosphoric acid, followed by filtration through a 0.5-μm Millipore filter and degassing) is pumped by means of an Orlita DMPAE 10.4 pump over a cation-exchange column (Nucleosil 5 SA 200/6/4, No. 715 300, Macherey, Nagel & Co., Düren, G.F.R., 150 bar, 1.5 ml/min) and the eluate is fed to a Metrohm EA 1096/2 flow-through cell. This cell is equipped with a three-electrode system (working electrode gold, EA 286/3; reference electrode silver–silver chloride with 3 *M* potassium chloride solution, EA 442; auxiliary electrode glassy carbon, EA 286/1), leading the signals to a Metrohm EA 611 amperometric detector. The polarization voltage is adjusted to +800 mV, the signal sensitivity is $12.5 \cdot 10^{-7}$ A for urine samples and $12.5 \cdot 10^{-8}$ A for plasma samples, and there is no need to damp the signal. A Servogor RE 511 recorder of sensitivity 1 V and paper advance speed 0.5 cm/min is used. For injecting the samples and standards a Hewlett-Packard 7671 A sampler, modified for HPLC, with a 20-μl loop, is used[17]. After five samples a standard is injected. The control of the analysis programme and the evaluation is performed with a laboratory data system (Hewlett-Packard 3353), the dilution factor of 3.3 for the samples being taken into account.

RESULTS AND DISCUSSION

Under the conditions used the retention times of D-penicillamine are 6 and 7.4 min and the detection limits are 0.05 μg/ml and 0.2 μg/ml for plasma and urine, respectively.

The coefficient of variation ($n = 10$) is 2.9%. The correlation between peak height and concentration of standard solutions, obtained without changing the sensitivity of the current measurement, is given in Table I.

TABLE I

RELATIONSHIP BETWEEN CONCENTRATION AND SIGNAL

Concentration (μg/ml)	*Peak height (mm) (average of 3 measurements)*
0.1	12
0.5	63
1.0	124
	$r = 0.9999$

Although the D-penicillamine in the plasma and urine samples prepared for analysis is stable for 8 h at 20°C (see Table II), the concentration decreases in native plasma in spite of the addition of 1% of Titriplex III (Table III).

The yield in the reduction of D-penicillamine disulphide to D-penicillamine[11] at a mercury electrode is 75–77% within 12 min.

The high selectivity and high sensitivity of the electrochemical detection of the SH group of D-penicillamine, separated chromatographically, permits its very rapid, automated determination without derivatization (Figs. 1 and 2).

It can be seen that urine samples contain larger amounts of detectable by-products than do deproteinized plasma samples, which is why for urine samples the

TABLE II

STABILITY OF D-PENICILLAMINE IN SAMPLES PREPARED FOR ANALYSIS

Time (h)	D-*Penicillamine (μg/ml)*	
	Plasma	*Urine*
0	0.70	1.14
1	0.70	1.14
2	0.68	1.11
3	0.69	1.16
4	0.72	1.17
5	0.70	1.14
6	0.72	1.14
7	0.71	1.14
8	0.70	1.12

TABLE III

STABILITY OF D-PENICILLAMINE IN PLASMA CONTAINING 1% OF TITRIPLEX III

Time (min)	D-*Penicillamine (μg/ml)*
0	2.9
5	2.7
10	2.5
20	2.3
30	2.1
60	1.7
120	1.1

eluent has been modified slightly to secure a better separation of different metabolites from D-penicillamine. Reduced glutathione and cysteine elute before D-penicillamine (Fig. 3).

Is is essential to use a solute similar to the eluent in order to avoid changing the milieu of the gold electrode. If deproteinization is effected by means of ethanol, the conversion at the electrode for D-penicillamine in the injected sample is reduced to 60%, so that this method of deproteinization consequently cannot be applied. During the analytical run a small decrease in conversion at the gold electrode and a reduction of signal is observed, but this decrease can be accounted for mathematically by injecting standards in a certain sequence. If the intensity of the signals decreases too much, the gold electrode must be polished with alumina (Metrohm). It is not necessary to polish the auxiliary electrode.

The detection limits of this method are equal to those of the method of Saetre and Rabenstein[11]. Increasing the injection volume to 100 μl reduces the detection limit by a factor of 4, but this can cause the accelerated formation of a film on the gold electrode with urine samples, which cannot be prevented by using a pre-column. Such a pre-column has proved not to be necessary, because even after the analysis of 1000

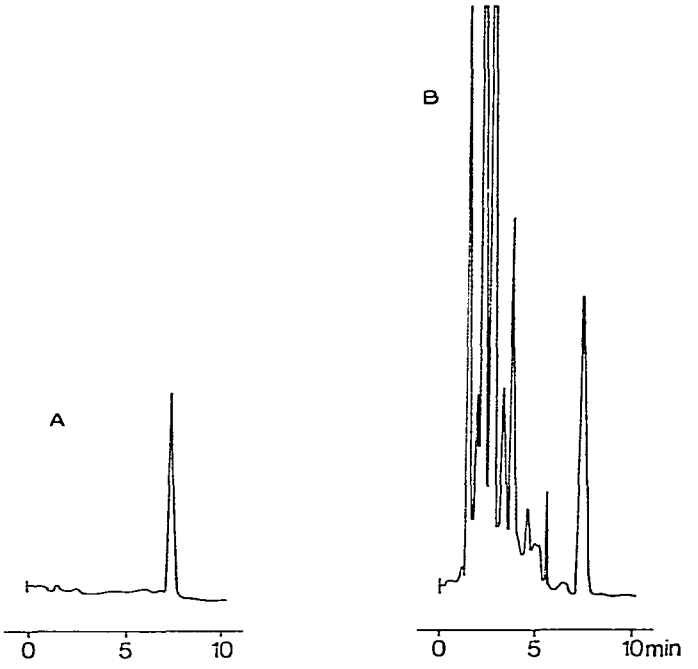

Fig. 1. Determination of D-penicillamine in urine. A, Standard (5 μg/ml); B, sample.

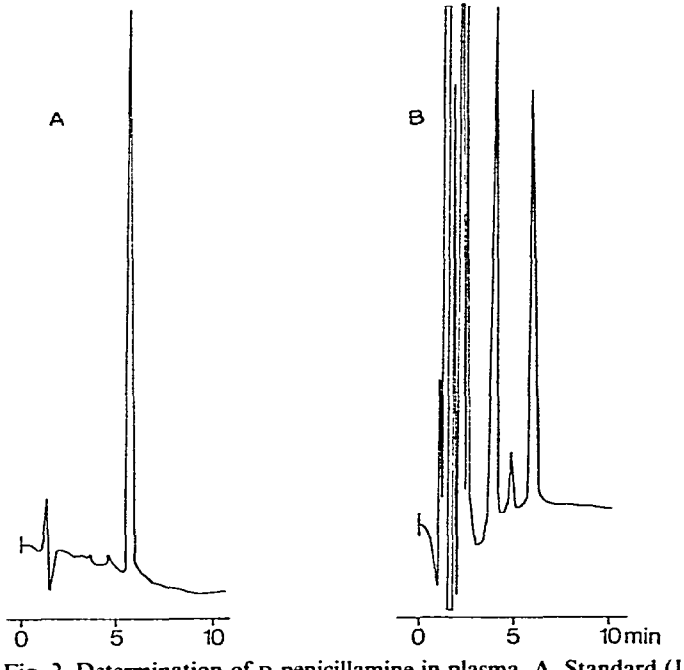

Fig. 2. Determination of D-penicillamine in plasma. A, Standard (1 μg/ml); B, sample.

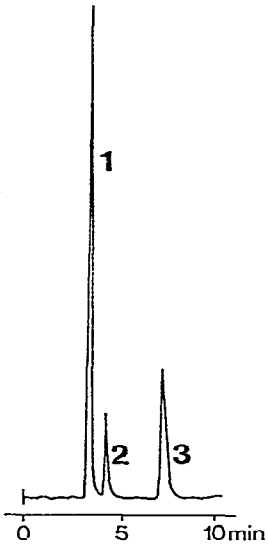

Fig. 3. Separation of L-cysteine (1), L-glutathione (2) and D-penicillamine (3), 1 μg/ml each; sensitivity $12.5 \cdot 10^{-8}$ A.

samples the column shows an unchanged separation power for the samples prepared in the manner described above.

Adjusting the detector to a higher sensitivity ($5 \cdot 10^{-8}$ A) results in baseline drift after a short equilibration time of the electrode, and high-frequency disturbances may also occur. For an automated run, for example overnight, it is advisable not to set a high sensitivity. It is recommended that the flow-through cell is screened.

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