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Note

Determination of D-penicillamine with an amino acid analyser using fluorescence detection

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D-Penicillamine, D-(−)-2-amino-3-mercapto-3-methylbutanoic acid, is a hydrolytic degradation product of penicillin with various modes of action (for reviews, see refs. 1—3). It interacts with collagen and elastin syntheses. It also reduces disulphide groups in tissues and forms mixed disulphides with other substances containing thiol groups. In addition, it has metal-chelating properties and various effects on prostanoid synthesis [2]. Its first successful therapeutic application was in the treatment of Wilson's disease, a copper storage disorder [4]. More recently, penicillamine has been used widely in the treatment of rheumatoid arthritis [5]. Other clinical indications recently reviewed by Crawhall et al. [3] are cystinuria, metal intoxication, chronic hepatitis, primary biliary cirrhosis and hyperbilirubinaemia.

The concentration of penicillamine in biological fluids has been difficult to

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measure. Several approaches have been described, based on colorimetry [6—8], thin-layer chromatography [9, 10] gas chromatography [11, 12], amino acid analysis [13—16], radioactivity [17—19], high-performance liquid chromatography [20—26] and high-voltage electrophoresis [27].

Fluorescence as a possible detection method for penicillamine was first proposed, albeit unexplored, by Crawhall et al. [3]. It has been used in connection with thin-layer chromatography and high-voltage electrophoresis by Fahey et al. [27], and combined with high-performance liquid chromatography by Lankmayr and co-workers [24, 25] and by Miners et al. [26].

Automatic amino acid analysis would be a suitable routine determination method, but in most programmes the separation of penicillamine from proline has proved unsuccessful. In the method described here, the problem can be overcome with the use of fluorescence detection, which leaves proline unreactive and hence the entire value obtained respresents the coeluting penicillamine.

EXPERIMENTAL

Urine samples were obtained, with informed consent, from eight patients suffering from rheumatoid arthritis. They received D-penicillamine in divided doses two to four times per day. At the beginning of the collection of the 24-h urine, 50 ml of 15% (w/v) sulphosalicylic acid, buffered with 70 ml/l of 3 *M* lithium hydroxide, was added to the collection bottle. This was done in order to keep the pH of the urine between 2 and 4 during the collection, to avoid loss of penicillamine [21]. After the total volume of urine had been measured, an aliquot was centrifuged at 300 *g* for 15 min at 4°C. The supernatants were stored at —20°C and 100-μl aliquots were used for penicillamine determinations within a week of collection of urine. The loss of penicillamine has been reported to be minimal over a period of six to eight days [21].

The analyses were made with a Kontron Chromakon 500 amino acid analyser (Zurich, Switzerland) equipped with a fluorescence accessory. The excitation wavelength was 350 nm, the emission wavelength 450 nm and the photomultiplier voltage 400—900 V. The column was 200 mm × 3.2 mm I.D. filled with Kontron AS 70 resin in lithium form [28].

The following buffers for the physiological fluid programme were used: Buffer 1: 5.05 g of lithium hydroxide, 17.4 g of citric acid, 70 ml of methanol and 9.9 ml of hydrochloric acid in 1 l of water, pH 2.60. Buffer 2: 5.45 g of lithium hydroxide, 17.4 g of citric acid, 50 ml of methanol and 7.5 ml of hydrochloric acid in 1 l of water, pH 3.20. Buffer 3: 8.4 g of lithium hydroxide, 17.4 g of citric acid and 11.9 ml of hydrochloric acid in 1 l of water, pH 3.60.

After filtration through a Millipore 0.22-μm filter, 2 ml of 30% (w/v) Brij 35 solution were added to each buffer solution. Buffer changes were made at 12 and 40 min, and the starting temperature of 37°C was changed to 54°C at 47 min. The column was regenerated for 10 min at 80°C with 0.4 *M* lithium hydroxide after each injection, and stabilized for 35 min with the first buffer. The fluorescence reagent was made according to Benson and Hare [29]. The flow-rate was 0.4 ml/h for both the buffer and the fluorescence reagent.

The pressure of the buffer pump was 100—130 bar and that of the reagent pump 10—20 bar.

All chemicals were of the highest purity available and they were obtained from Merck (Darmstadt, F.R.G.) or Sigma (St. Louis, MO, U.S.A.). The detection limit and the linearity range were determined with reference D-penicillamine (free base) obtained from Medica Pharmaceutical Co. (Helsinki, Finland).

RESULTS

Typical chromatograms of a conventional and a D-penicillamine-spiked reference mixture are shown in Fig. 1. The limit of detection is 25 μmol/l (3.73 mg/l) (Fig. 2), and the determination is linear over the range 25 μmol/l to 10 mmol/l (3.73—1490 mg/l). The use of higher values of photomultiplier voltage is hampered by the noise in the baseline. In Table I, the amounts of

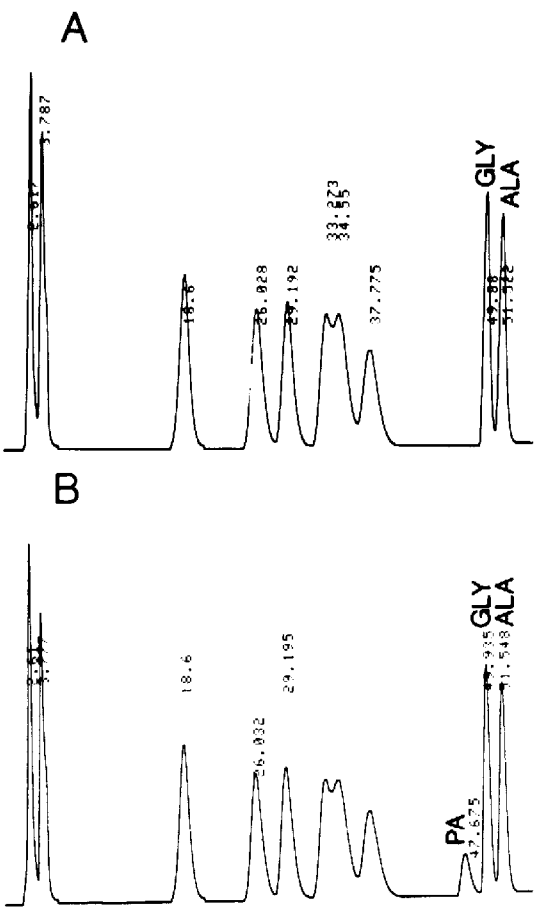

Fig. 1. (A) The beginning of the chromatogram from the amino acid standard "Physiological A/N" (Pierce, Rotterdam, The Netherlands). (B) The same chromatogram as in A, but now with 1 mmol/l D-penicillamine added. Sensitivity of the detector: 400 V. Peaks: PA = D-pencillamine; GLY = glycine; ALA = alanine.

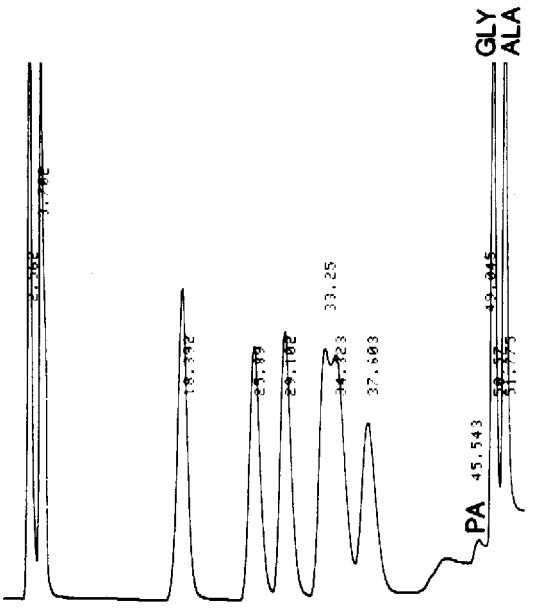

Fig. 2. Determination of the detection limit. An aliquot of 25 μmol/l D-penicillamine gives a reproducible peak. Sensitivity of the detector: 600 V. Peaks: PA = D-penicillamine; GLY = glycine; ALA = alanine.

TABLE I

AMOUNT OF PENICILLAMINE IN URINE OF PATIENTS RECEIVING D-PENICILLAMINE IN DIVIDED DOSES TWO TO FOUR TIMES PER DAY

Patient	Daily dosage (mg)	Amount in urine	
		mg per 24 h	μmol per 24 h
A.V.	300	8.22	55.1
K.L.	450	3.55	23.8
M.K.	600	6.55	43.9
M.S.	450	12.14	81.4
T.L.	600	7.61	51.0
R.P.	450	6.55	43.9
K.B.	450	6.40	42.9
R.G.	450	2.51	16.8

penicillamine excreted into urine in 24 h from patients receiving this drug are listed. A chromatogram of a urine sample from a patient with rheumatoid arthritis receiving 600 mg of D-penicillamine per day is shown in Fig. 3.

DISCUSSION

In the ninhydrin reaction, commonly used with amino acid analysis, the colour yield of the free thiol of D-penicillamine is poor. Hsiung et al. [15] found it unsuitable for measurements without prior formation of a derivative. They used S-carboxymethyl or N-ethylmaleimide derivatives, which block the thiol groups originating from oxidized and reduced forms of the thiols and from mixed disulphides.

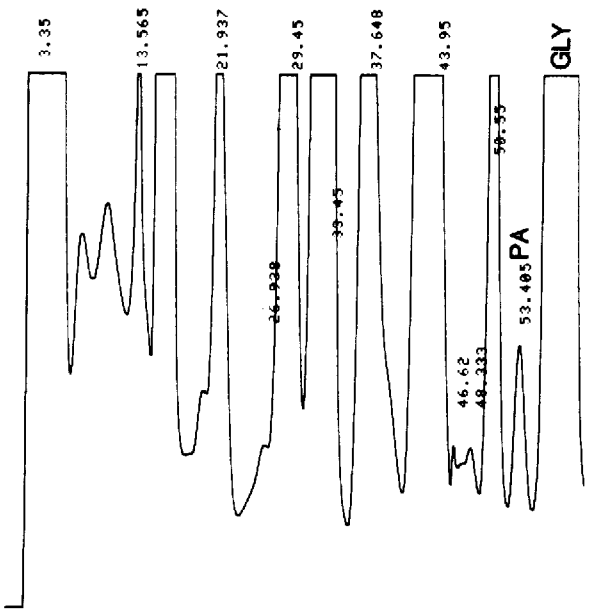

Fig. 3. Urine sample from a patient with rheumatoid arthritis receiving 600 mg of D-penicillarmine per day. Sensitivity of teh detector: 600 V. Peaks: PA = D-penicillamine; GLY = glycine.

Borner [13] converted penicillamine and penicillamine disulphide to penicillaminic acid (3,3-dimethylcysteic acid) with performic acid and analysed the derivative on an anion-exchange column (Dowex 1X8) with ninhydrin detection. Performic acid oxidation was also used by Muijsers et al. [16], with a subsequent separation, which separation we were unable to repeat on Aminex A-25 resin in a Beckman Multichrom M amino acid analyser.

Lankmayr and co-workers [24, 25] investigated the selective reaction of thiol compounds with 5-dimethylaminonaphthalene-1-sulphonylaziridine. The derivatives were stable and gave high fluorescence yields. Having found this procedure difficult to reproduce, Miners et al. [26] developed a method based on the fluorescence of N-[*p*-(2-benzoxazolyl)phenyl]maleimide derivative. The reaction of *o*-phthalaldehyde, frequently used in the detection of primary amines, proceeds rapidly enough to prevent penicillamine auto-oxidation.

In older amino acid analysers with long cycles, the reduced penicillamine was incompletely resolved from glycine [13] or coeluted with alanine [14]. However, a good separation can be achieved with a modern amino acid analyser. The advantage of the fluorescence detection method lies in the fact that it eliminates the presence of proline, which is apt to coelute with penicillamine. Furthermore, the metabolites of D-penicillamine have different retention times and do not interfere with this method. The present study has been carried out with the column and programme for physiological amino acids in biological fluids (Li^+ system), but the Na^+ system might be equally convenient for the determination of penicillamine [28].

The results obtained from the urine samples in this study are in agreement with previous reports [20, 23]. No correlation was found between dosage and

urinary excretion of penicillamine. This method is also amenable for plasma samples, provided that they are prepared according to Bergstrom et al. [21]. It has been proposed [18] that the levels of penicillamine in the plasma and urine of patients should be monitored to provide optimal treatment with minimal side-effects. Moreover, amino acid analysis gives additional information besides the penicillamine level, viz. the concentration of histidine in plasma [30]. The change in plasma histidine caused by penicillamine may be a side-effect or reflect a specific action of the drug in disease pathogenesis [31].

In summary, a selective and sensitive method for the determination of penicillamine has been described. Attention to the preparing, storage and transport of the samples, as well as a prompt analysis, is emphasized in this connection.

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