A simple colorimetric method for the estimation of D(—)-penicillamine in plasma

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The mode of action of penicillamine in rheumatoid arthritis is still unknown and investigations using animal models could be aided by the ability to measure plasma penicillamine concentrations. No colorimetric method that can be used routinely for measuring penicillamine in plasma is available. The colorimetric method of Pal (1959) is unreliable since it employs heat treatment of plasma, a procedure which we have found will degrade penicillamine in plasma. More complex methods using chromatographic (Borner 1965), and immunological procedures (Assem & Vickers 1974) have been reported. Recently Saetre & Rabenstein (1978) described a sensitive method using high pressure liquid chromatography in conjunction with a specialized detection unit for thiol groups.

We have devised a simple colorimetric method based on the use of Ellman's reagent, 5,5'dithiobis-2(nitrobenzoic acid), DTNB (Ellman 1959). Although the method is not as sensitive as the h.p.l.c. technique it is sufficiently sensitive to allow kinetic studies in animal models of rheumatoid arthritis and does not require specialized apparatus. The method has been used to measure the plasma concentration of penicillamine in the rabbit after administration of the drug by various routes. The plasma concentration of penicillamine in animals has not previously been reported.

Male and female Old English rabbits, 2·0–3·0 kg, were used. For the studies using parenteral administration of penicillamine, animals were maintained throughout the experiment under halothane/nitrous oxide/oxygen anaesthesia to allow multiple blood sampling from a cannulated carotid artery. The oral study used conscious animals, blood samples being taken by cardiac puncture under anaesthesia from three animals, at each time interval. The method was further evaluated using plasma from rheumatoid arthritis patients receiving oral penicillamine therapy.

Preparation of plasma. Blood was collected into 1/10 of its final volume of Tris-EDTA(Tris-25mM, EDTA-100mM, NaCl 120mM, KCl 5mM, adjusted to pH 7·4) cooled in ice, and centrifuged at 800 *g* for 10 min. Plasma aliquots of 1·1 ml were mixed with 0·1 ml 3M-HCl, immediately frozen in a solid CO_2/methanol bath, and freeze-dried overnight. Plasma samples containing penicillamine are stable when freeze dried but not when frozen at —20 °C. Ethanol (1·5 ml) was added to the freeze-dried plasma samples, the suspensions sonicated for 5–10 s, and then vortex mixed for 30 s. The samples were centrifuged at 800 *g* for 10 min and ethanol supernatant (1 ml) removed.

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Reaction with DTNB. The ethanol supernatant was added to an equal volume of 0·45 M Tris-HCl, pH 8·2 and mixed with 20 μl of DTNB (10mM in 0·1 M-phosphate buffer, pH 7·0). Diethyl ether (4 ml) was added, the tubes shaken for 10 min, and then centrifuged for 10 min at 800 *g*. The upper ethanol–ether layer was discarded, and the absorbance of the lower aqueous layer measured at 412 nm. Plasma samples containing 5–40 μg added penicillamine were carried through the procedure with each assay. A plasma sample was removed from each animal before penicillamine was given to serve as control. When plasma from rheumatoid patients was used, a control sample was obtained as follows. Each sample was divided into two and one aliquot heated to 50 °C for 1 h with vortex mixing at 5 min intervals. This procedure completely destroyed the ability of any penicillamine in the plasma to react with DTNB, presumably by oxidizing the penicillamine to a disulphide. The heated sample served as control.

A calibration curve for penicillamine in a pooled sample of rabbit plasma was linear to 50 μg ml^{-1} penicillamine and concentrations down to 2 μg ml^{-1} could be detected (1·0 Absorbance unit ≃50 μg). The method gave a recovery of 66% (4) (mean with s.d., n = 4) of penicillamine added to plasma. It was

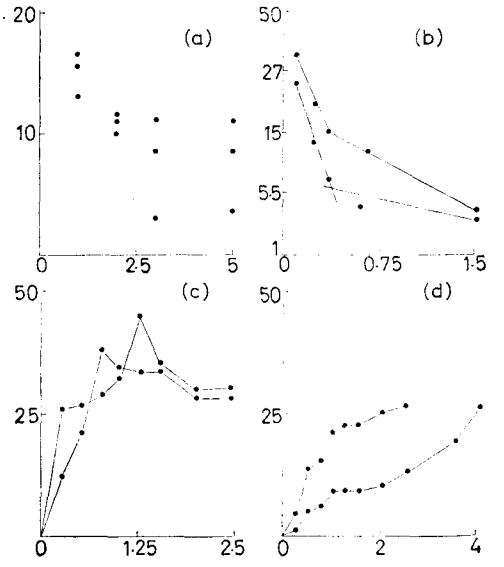

FIG. 1. Plasma concentration of penicillamine in rabbits administered (a) 50 mg kg^{-1} orally, (b) 10 mg kg^{-1} intravenously, (c) 25 mg kg^{-1} intraperitoneally, (d) 25 mg kg^{-1} subcutaneously. Results are for individual animals. Ordinates: μg penicillamine ml^{-1} plasma. Abscissae: time (h) post dose.

essential to convert the penicillamine in plasma to the hydrochloride before freeze-drying since penicillamine base is only slightly soluble in ethanol. It was also necessary to use EDTA as anticoagulant, since penicillamine could not be detected in ethanol extracts if either heparin or citrate was present as anticoagulant or if penicillamine was added to serum. Although Saetre & Rabenstein (1978) used EDTA they did not comment on its necessity. The requirement for EDTA implies that penicillamine circulates in plasma complexed with a cation such as copper. Penicillamine-copper complex has been reported to possess activity as a superoxide dismutator (Lengfelder & Elstner 1978), and a deficiency of superoxide dismutase activity in rheumatoid arthritis has been observed (Rister et al 1978). The therapeutic activity of penicillamine may arise from the ability of its copper complex to protect cells from superoxide ions generated in phagocytosis.

The plasma concentrations of penicillamine administered by four different routes to rabbits are shown in Fig. 1. Oral absorption was rapid (Fig. 1a) and the plasma concentration fell to a steady value 3 h after administration. When penicillamine was given intravenously removal from plasma followed a biphasic pattern (Fig. 1b). An initial rapid phase occurred with a $T_{1/2}$ of 21 min followed by a slower phase with a $T_{1/2}$ of 55 min. Penicillamine was rapidly absorbed from the peritoneal cavity (Fig. 1c). Absorption was slowest following subcutaneous administration; peak plasma concentrations had not been reached by 4 h.

When rabbits were given 20 mg kg^{-1} i.v. daily doses of penicillamine for 21 days, no detectable penicillamine was present in the plasma 25 h after the final dose.

The plasma from six rheumatoid arthritis patients receiving chronic penicillamine therapy was also analysed by the method. Two h after they had taken 125 mg penicillamine the penicillamine plasma concentrations were less than 2 $\mu g\ ml^{-1}$. Saetre & Rabenstein (1978) reported values of $1{\cdot}67 \pm 0{\cdot}90\ \mu g\ ml^{-1}$ in a group of six rheumatoid patients receiving 250 mg penicillamine three times daily. Only one other report of plasma penicillamine concentrations in man has been recorded. Assem (1974), using a radioimmune assay, found 8–10 $\mu g\ ml^{-1}$ in a single subject 2–3 h after a single oral dose of 250 mg penicillamine. This higher value for penicillamine may be a consequence of the radioimmune assay detecting metabolites, such as the disulphide of penicillamine. Alternatively, the chronic administration of penicillamine to patients may induce a more rapid metabolic destruction of the drug.

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Polarographic assay of glyceryl trinitrate by an internal standardization method

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Studies by Flann (1969) and Thoma & Groening (1975) have amply demonstrated the application of a polarographic method which is well suited to single dosage form assays. Although Flann (1969) reported good reproducibility of the (glyceryl trinitrate concentration/diffusion current) ratio over a period of 7 weeks in a thermostatted system, it is common practice to make use of an internal standard to overcome the effect of variation in instrumental factors, and it was decided to test the reproducibility of a non-thermostatted system over a long period during which the instrument would be used intermittently by other workers, mainly students. Because the polarographic reduction of glyceryl trinitrate is an irreversible process (Flann 1969) it was considered desirable to use a standard chemically similar to it.

Polarographic solvent: 800 ml n-propanol, 100 ml 1·0 M tetramethylammonium chloride, 50 ml 0·2 M ammonium chloride, 50 ml 0·2 M ammonium hydroxide. Methyl nitrate (BDH Poole U.K.), 0·2 mg ml^{-1} in acetone, was used as the internal standard. The glyceryl trinitrate standard was extracted from mannitol-based tablets (A.P.S., Cleckheaton, U.K.) by dry ether. Evaporation under vacuum of the ethereal extract yielded an oil which gave a single peak on g.l.c. analysis (Rosseel & Bogaert 1972) which was dissolved in acetone for the polarographic assays. Polarograph: Cambridge Instruments Recording Polarograph. 1 ml aliquots of glycerol trinitrate in acetone and methyl nitrate internal standard solutions were added to 3 ml of polarographic solvent in the polarograph cell and de-oxygenated by bubbling nitrogen for 5 min. The polarograms were recorded between 0 and —2·2 V using a dropping mercury cathode and mercury anode, and the diffusion