Br. J. clin. Pharmac. (1990), **30**, 511–517

D-penicillamine and D-penicillamine-protein disulphide in plasma and synovial fluid of patients with rheumatoid arthritis

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1 The plasma pharmacokinetics of D-penicillamine (D-pen) and D-penicillamine-albumin disulphide (D-pen-alb) were examined over a dosage interval in six patients with rheumatoid arthritis. In two of these, 24 h synovial fluid profiles of D-pen and D-pen-alb were also obtained.

2 D-pen was undetectable in plasma at the beginning of the study. The peak concentration (5.4 ± 1.2 μM) occurred at between 45 min and 2 h and the mean elimination half-life was 0.6 h. D-pen-alb, however, was present at a mean plasma concentration of 19.1 μM prior to dosage, peaked at 26.2 μM and was eliminated with a half-life of 40 h.

3 D-pen concentrations in synovial fluid rose more slowly and peaked lower than in plasma. D-pen-alb was present in synovial fluid of the patients at 50.1% and 83.6%, respectively, of the simultaneous plasma concentration prior to dosage. Concentrations varied during the study interval, corresponding to changes in plasma concentrations.

4 These results demonstrate that D-pen forms stable conjugates with protein in treated patients. The presence of D-pen-alb in relatively high concentrations throughout the dosage interval contrasts with the low concentrations and rapid elimination of D-pen. Both D-pen and D-pen-alb were also shown to be present at the putative site of drug action (the inflamed synovial joint) in concentrations lower than those in plasma.

Keywords D-penicillamine protein binding rheumatoid arthritis blood synovial fluid

Introduction

D-Penicillamine (D-pen) is a thiol which modifies the course of rheumatoid arthritis in some patients. Neither the mechanism of action nor the active form of the drug are known with certainty. Investigation has been hampered by the unavailability of an animal model of D-pen-responsive rheumatoid arthritis and by ignorance of the pharmacokinetics of the drug. To date, adequate pharmacokinetic studies have been reported only of D-pen (Bergstrom *et al.*, 1981; Brooks *et al.*, 1984; Kukovetz *et al.*, 1983) and its low molecular weight metabolites in plasma and urine (Carruthers *et al.*, 1984; Kyogoku *et al.*, 1982; Perrett, 1981). The concentrations in the synovial joint, the putative site of action, have not been measured. There have been no studies on the pharmacokinetics of the protein conjugate of D-pen, either in blood or synovial fluid, despite recognition of the quantitative importance of this metabolite (Gibbs & Walshe,

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1971) and its possible role in D-pen action (Binderup & Arrigoni-Martelli, 1979; Watanabe *et al.*, 1986). This conjugate has the characteristics of a disulphide linking D-pen and plasma albumin (Gibbs & Walshe, 1971; Joyce & Wade, 1988) and is therefore similar to the D-pen-albumin (D-pen-alb) disulphides formed in rats (Joyce *et al.*, 1989) and *in vitro* (Joyce & Wade, 1988).

Because the forms and concentrations of D-pen at its site of action are unknown, much *in vitro* and animal experimentation has been done using arbitrary dosage of the parent drug. Ambiguous or contradictory results have often emerged. The aim of this study is to provide information on the concentrations of D-pen and D-pen-alb in the plasma and synovial fluid of patients with rheumatoid arthritis. These data may then be used to guide investigations into the mode of action of D-pen in rheumatoid arthritis.

Methods

Six patients (five females, one male), aged between 24 and 71 years with rheumatoid arthritis (American Rheumatism Association criteria: Arnett *et al.*, 1988) of over 5 years duration were studied. The patients were free of other significant disease detectable by history and physical examination and had normal blood leucocyte and platelet counts, plasma concentrations of liver enzymes, urinalyses and plasma creatinine concentrations. Two had mild normochromic, normocytic anaemia (haemoglobin concentrations of 11.3 and 11.7 g dl^{-1}, respectively). All patients took non-steroidal anti-inflammatory drugs chronically, but none had received systemic corticosteroid therapy or other slow-acting antirheumatic drugs in the 6 months preceding the study. The patients had been taking non-enteric coated D-pen (250 mg daily: Dista Products) for a minimum of 3 months prior to study. Rheumatoid arthritis had partially remitted in three patients during therapy and had not altered objectively in two others. The sixth patient had remitted on a dose of 500 mg daily, but disease activity had increased at the time of study, which was 8 months after dosage reduction. None had experienced a significant reaction to D-pen at any time. The concentrations of D-pen and D-pen-alb in plasma and synovial fluid were measured simultaneously in two of the patients, aged 68 and 71 years. Both were females with long-standing, classical rheumatoid arthritis and large knee effusions. These joints had not been aspirated or injected with corticosteroid in the preceding 6 months. One of these two patients had been treated with D-pen for over 1 year and had remitted partially. The other had been treated for 3 months without objective signs of remission. Informed consent was obtained from each patient and the protocol was approved by the Ethics Commitee of St Vincent's Hospital and the Committee on Experimentation involving Human Subjects of the University of New South Wales.

Patients presented, fasting, on the first morning of study and a Teflon intravenous catheter (Jelco ™) was inserted in a forearm vein for blood sampling up to 6 h. An initial blood sample (10 ml) was collected into a sterile tube containing EDTA (Vacutainer ™) and mixed immediately. A D-pen tablet (250 mg, Dista) was then given orally with 200 ml of water. Further blood samples were collected at 0.5, 1, 2, 4, 6, 8 and 24 h. Additional blood specimens were collected from some patients at 15 min ($n = 4$), 45 min ($n = 4$) and 12 h ($n = 3$). Synovial fluid samples (3 ml) were collected from the two patients prior to D-pen ingestion and at 0.5, 1 (one patient), 2, 4, 6, 8, 12 and 24 h. A medial retropatellar approach, bupivacaine local anaesthesia and aseptic technique were used. Samples were placed in sterile tubes containing EDTA (Vacutainer ™) and mixed immediately.

Blood specimens were separated by centrifugation (1700 *g* for 10 min at 4° C) immediately after collection, to limit *in vitro* oxidation of D-pen (Bergstrom *et al.*, 1980). Aliquots of plasma and synovial fluid (1 ml) were added to polypropylene tubes containing 400 μl 18% w/v trichloracetic acid (TCA), allowed to stand at 0° C for 10 min and then centrifuged (2000 *g* for 5 min at room temperature) to yield clear supernatants and protein-containing precipitates. These were separated and supernatant was stored at −20° C until assay within a week. Protein precipitates were washed and assayed for D-pen-alb as described previously (Joyce & Wade, 1988). Briefly, washed and dried protein precipitates were resuspended in Tris buffer under anoxic conditions, reduced with $NaBH_4$ and reprecipitated with $HClO_4$. D-pen was then assayed in the supernatant, as below. This assay is sensitive to 1.2 μM D-pen-alb in plasma and has an intra-assay coefficient of variation of 4.6% at 23.9 μM in plasma and 5.6% in pooled synovial fluid.

H.p.l.c. separation and gold/mercury electrochemical detection (Drummer *et al.*, 1986) were used to assay the D-pen in plasma and synovial fluid supernatants and the D-pen liberated from D-pen-alb. A Varian 5020 h.p.l.c., including a single-piston pump and system controller was used. Samples were injected using a Kortec 65A Autosampler (ETP-Oxford Pty Ltd, Ermington,

N.S.W., Australia) equipped with either a 20 μl or a 50 μl injector loop. The stationary phase was a 10 cm × 4.6 mm Rainin 'Short One' C18 column (3 μm particle size), maintained at room temperature. Monochloroacetic acid/NaOH buffer (BDH, 0.1 M, pH 3.0) with 1 g l^{-1} heptane sulphonic acid (BDH) and 6% v/v acetonitrile (Mallinckrodt, Putney, NSW, Australia) was used as the mobile phase and all runs were isocratic at a flow rate of 0.6 ml min^{-1}. Mobile phase was deoxygenated by boiling under reflux conditions and bubbling it with high purity helium (CIG, Australia) prior to and throughout the run. A small amount of L-cysteine (BDH) was added to samples and standards (10 μl of 1 mM aqueous solution added to 200 μl of sample or standard) to assist in maintaining the sensitivity of the assay, presumably by acting as an antioxidant. It eluted with the solvent front.

A BAS LC-4B/19 amperometric detector with BAS TL-6A Hg/Au working electrode and a glassy carbon auxiliary electrode (Bioanalytical Systems Inc.) was linked to the column eluate line. The mercury surface of the working electrode was replaced when assay sensitivity exceeded 1 μM. The working electrode potential was maintained at +150 mV with respect to a silver/silver chloride reference electrode. The detector sensitivity was 50 nA per volt and output was integrated and plotted by a Hewlett-Packard model 3390A integrator. D-pen eluted at 4.66 min and was identified and measured by comparison with authentic standards. Four point standard curves were run before and after each group of twenty samples to compensate for variation in detector sensitivity. The coefficient of variation of the assay for D-pen in synovial fluid was 4.5% at 1.0 μM and 3.6% at 10.0 μM, with a sensitivity of 0.5 μM. The plasma D-pen assay performed comparably.

Plasma concentration-time data were fitted by a two-compartment open model (Figure 1), using a non-linear, extended least-square procedure (MK-Model: Elsevier-Biosoft, Cambridge, UK). Rate constants for D-pen absorption (k_{01}), transformation to D-pen-alb (k_{12}) and elimination by other pathways were estimated (k_{10}). The rate constant for elimination of D-pen-alb was also estimated (k_{20}). The bioavailability (F) of D-pen was estimated from the AUC for D-pen for each subject and published estimates of the volume of distribution of D-pen ($V_{D\text{-pen}}$), utilising the relationship:

$$F = V_{D\text{-pen}} \times AUC_{D\text{-pen}} \times k/D_{D\text{-pen}}$$

where k is the estimated terminal elimination rate constant for D-pen, calculated from log-linear regression of concentration-time data

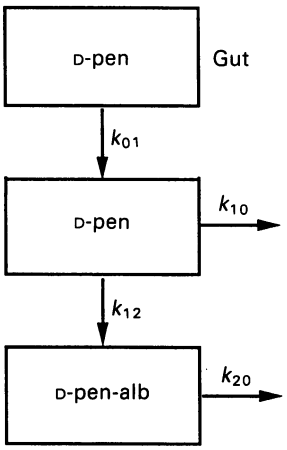

Figure 1 Model for D-pen absorption, metabolism to D-pen-alb, elimination as D-pen-alb and elimination by other routes.

between 2 and 8 h (or 12 h where the data were available) and $D_{D\text{-pen}}$ is the dose (μmol). $V_{D\text{-pen}}$ was taken as 1.54 l kg^{-1} (Kukovetz *et al.*, 1983). The volume of distribution of D-pen-alb ($V_{D\text{-pen-alb}}$) was assumed to equal the volume of distribution of plasma albumin. This was calculated from the data of Schultze & Heremans (1966), adjusted for the age, sex and bodyweight of our patients according to the method of Dagher *et al.* (1965). The amount of D-pen converted to D-pen-alb ($D_{D\text{-pen-alb}}$) during each dosage interval was estimated using the relationship:

$$D_{D\text{-pen-alb}} = V_{D\text{-pen}} \times AUC_{D\text{-pen}} \times k_{12}$$

Results are expressed as mean ± s.e. mean.

Results

D-pen was not detected in the pre-dose plasma sample nor the 24 h sample from any patient. Peak concentrations occurred at between 45 min and 2 h and the mean peak concentration was 5.4 ± 1.2 μM (Figure 2). Bioavailability was calculated to be 0.44 ± 0.10. The mean apparent absorption rate constant was 0.38 h^{-1} and the elimination rate constant for pathways other than protein conjugation was 1.13 ± 0.21 h^{-1}. Concentrations of D-pen and D-pen-alb predicted by the model closely approximated observed values in all cases. Data fitting was not improved by incorporation of a time lag for D-pen absorption in the model. D-pen-alb was present in plasma throughout the dosage interval, and rose from a mean concentration of 19.1 ± 3.0 μM, pre-dose, to a peak of 26.2 ± 4.0 μM. The mean concentration at 24 h was 20.9 ± 3.0 μM. The rate

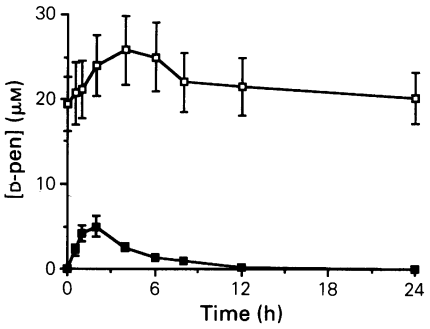

Figure 2 Concentrations of D-pen (■) and D-pen-alb (□) in plasma of patients with rheumatoid arthritis during a dosage interval. Patients were treated chronically with D-pen, 250 mg daily.

constant for D-pen elimination by conjugation to protein was $0.029 \pm 0.006\ h^{-1}$, and the conjugate represented 9.99 ± 2.49 mg or 9% of the bioavailable D-pen. The rate constant for elimination of D-pen-alb was $0.017 \pm 0.005\ h^{-1}$. These values correspond to mean half-lives of 0.59 h for D-pen and 40 h for D-pen-alb.

D-pen was undetectable in synovial fluid prior to dosing and peak concentrations were lower and later in synovial fluid than in plasma. Peak concentrations of 2.4 μM and 1.5 μM were found in synovial fluid at 4 and 6 h after dosing in the two patients (Figure 3). These compared with peak plasma concentrations of 4 μM at 1–2 h and 3.9 μM at 2 h respectively. Equilibrium between plasma and synovial fluid concentrations of D-pen was achieved at between 4 and 6 h in patient 1 and between 8 and 12 h in patient 2. Thereafter, synovial fluid concentrations exceeded plasma concentrations until both became negligible. The initial concentrations of D-pen-alb in synovial fluid were 22.0 μM and 10.45 μM, which corresponded to plasma D-pen-alb concentrations of 26.3 μM and 20.84 μM, respectively. The peak concentrations of D-pen-alb were 29.6 μM at 6 h and 12.1 μM at 4 to 6 h, respectively. These times were similar to the times of the plasma peaks. Final concentrations of D-pen-alb were 21.6 μM and 8.5 μM in synovial fluid and 28.8 μM and 19.5 μM respectively in plasma in the two patients.

Discussion

D-pen is present in plasma and synovial fluid at low concentrations for approximately 8 h after a 250 mg dose in patients with rheumatoid arthritis. Values for its bioavailability, absorption rate and elimination rate were similar to those reported previously in normal subjects and patients with rheumatoid arthritis (Bergstrom *et al.*, 1981;

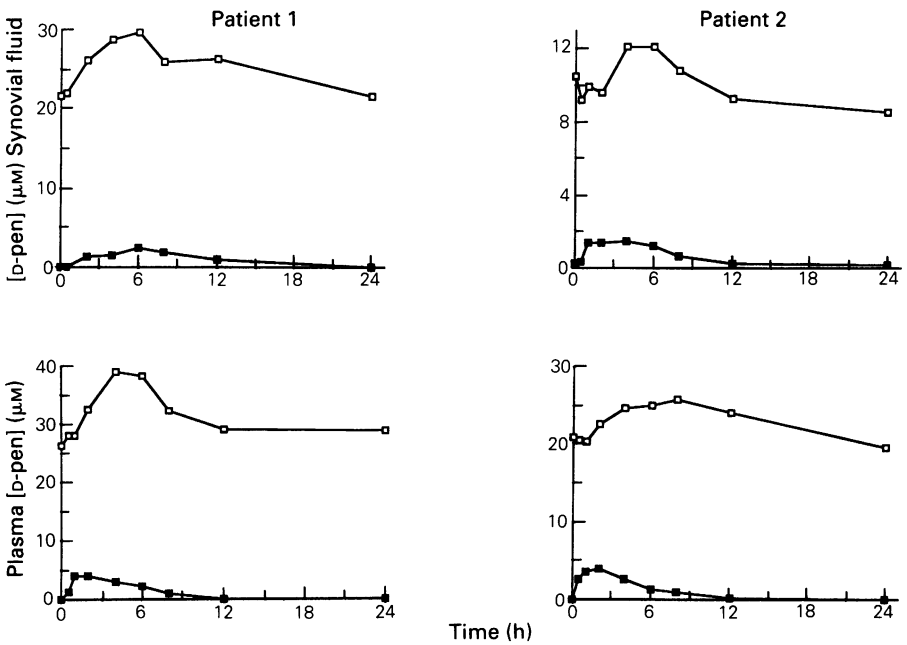

Figure 3 Concentrations of D-pen (■) and D-pen-alb (□) during a dosage interval in plasma and synovial fluid of two patients with rheumatoid arthritis.

Kukovetz *et al.*, 1983; Kyogoku *et al.*, 1982). Although most D-pen is eliminated through conversion to low molecular weight metabolites, such as disulphides and S-methyl-D-pen and through renal excretion (Perrett, 1981), a significant proportion (9%) is also converted to D-pen-alb. D-pen-alb forms when D-pen is incubated with plasma *in vitro* (Joyce & Wade, 1988), so circulating plasma is likely to be the site of formation *in vivo*. The long half-life of D-pen-alb (40 h) contrasts with the rapid elimination of D-pen itself (half-life of 0.6 h). The pharmacokinetic model which was used to derive the half-life of D-pen-alb does not distinguish between distribution and elimination, so the true elimination half-life may be longer. The short sampling period, relative to the long half-life of D-pen-alb, may also lead to underestimation. Elimination of D-pen-alb presumably occurs through slow reduction of the disulphide linkage between albumin and D-pen, analogously to the *in vivo* reduction of low molecular weight disulphides of D-pen (Bourke *et al.*, 1984). Slow elimination permits accumulation of the albumin conjugate, explaining the relatively high concentrations pre-dose. The mean pre-dose D-pen-alb concentration was over 3.5 times greater than the mean peak D-pen concentration and therefore constituted an appreciable pool of available D-pen, if released by disulphide reduction. Slow release of this pool partially explains the presence of D-pen metabolites in the urine of chronically-treated patients long after the cessation of therapy (Perrett, 1981; Wei & Sass-Kortsak, 1970). Sequestration of D-pen in protein conjugates also partially explains incomplete recovery of D-pen during short-term balance studies (Perrett, 1981). Disulphides formed between D-pen and tissue protein may also exist, contributing to the delayed renal excretion of D-pen metabolites. Concentrations in synovial fluid are important because they generally approximate concentrations in synovium (Gaucher *et al.*, 1983; Javala *et al.*, 1977; Soren, 1977), the putative site of action of D-pen. Peak concentrations of D-pen were reached later in synovial fluid than in plasma and were lower. This is consistent with passive diffusion. Passive diffusion accounts for the passage of D-pen across other biological barriers (Lodemann, 1981; Perrett, 1981; Wass & Evered, 1970). D-pen is hydrophilic, so transynovial exchange is likely to be slower than the rapid transynovial exchange which occurs with highly lipophilic compounds (Simkin & Pizzorno, 1974).

D-pen-alb is expected to diffuse into synovial fluid at a similar rate to plasma albumin (Brown *et al.*, 1969; Simkin & Pizzorno, 1974). The clearance of plasma albumin into knee effusions in patients with rheumatoid arthritis (0.021 ± 0.003 ml min^{-1}: Simkin & Pizzorno, 1974) would account for 5.04 ml and 7.56 ml of plasma entering the joints by the times peak concentrations were reached at 4 h and 6 h, respectively, in the two patients. This largely explains the rise in synovial fluid D-pen-alb concentration in the second patient and partially explains the rise in the first. D-pen-alb may also form within the joint, but this could not be measured in the presence of transfer to and from plasma. D-pen-alb redistribution from the synovial fluid to the systemic circulation is presumably through synovial lymphatics, as occurs for albumin (Bauer *et al.*, 1933).

The mechanism of action of D-pen is unknown. Low concentrations are present in synovial fluid and, therefore, are available for interaction with oxygen-derived free radicals (Lipsky, 1984; Staite & Zoschke, 1984), collagen (Patzschke & Wegner, 1977), cartilage and inflammatory cells in the fluid and synovium. Concentrations would be proportionately higher in patients taking higher doses, since elimination obeys linear pharmacokinetics (Brooks *et al.*, 1984). However, the low concentrations of D-pen in plasma and synovial fluid and their rapid elimination contrast with the conditions under which the drug is usually studied. Conclusions on the mode of action of D-pen often have been drawn from experimental systems exposed to concentrations of drug which could never be achieved in the plasma or synovial fluid of patients taking anti-rheumatic doses of the drug.

Research has concentrated on the effects of D-pen on model systems, although it is not known whether it is the active form of the drug. The high concentrations and slow elimination of D-pen-alb contrast with the kinetics of the parent drug and indicate a possible means of inducing a stable modification in inflammatory cell function. D-pen-protein conjugates have been demonstrated on the surfaces of monocytes and macrophages incubated *in vitro* with D-pen-alb and D-pen, respectively (Binderup & Arrigoni-Martelli, 1979; Watanabe *et al.*, 1986). They may be responsible for the alterations in monocyte behaviour seen in D-pen-treated rheumatoid arthritis patients (Hurst *et al.*, 1986; McKeown *et al.*, 1984). D-pen-alb or other D-pen-protein conjugates may therefore contribute to the anti-rheumatic action of D-pen.

This work was supported by a Project Grant from the National Health and Medical Research Council of Australia.

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(*Received 13 March 1990, accepted 23 May 1990*)