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ASSAY FOR D-PENICILLAMINE–PROTEIN CONJUGATE IN HUMAN PLASMA UTILISING CHEMICAL REDUCTION FOLLOWED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH GOLD/MERCURY ELECTROCHEMICAL DETECTION

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SUMMARY

D-Penicillamine (D-pen), a thiol with antirheumatic activity, forms a mixed disulphide with albumin in vivo. This conjugate is important in the pharmacokinetics and possibly the mode of action of D-pen. An assay was devised for D-pen–albumin disulphide, based on separation from plasma by acid precipitation followed by quantitative reduction with sodium borohydride in an anoxic environment. The liberated D-pen was then assayed by high-performance liquid chromatography with gold/mercury electrochemical detection. The assay was sensitive to 1.2-μM D-pen–albumin disulphide (signal-to-noise ratio > 2), absolute recovery was 92.7% and intra-assay coefficient of variation was 4.6% in human plasma. This technique also may be useful for quantitating protein conjugates of other thiols.

INTRODUCTION

A stable conjugate between D-penicillamine (D-pen) and plasma protein forms during the oxidation of D-pen in vitro [1] and during treatment of rats and humans [2–4]. This conjugate has the characteristics of a disulphide [5] and albumin has been identified as the major protein involved [3]. Reduced sulphydryl groups are normally present on plasma albumin at a concentration of approximately 700 μM and these are presumed to be available for disulphide binding to D-pen [6]. D-pen–albumin disulphide appears to be important both in the pharmacokinetics and the mode of action of D-pen. It may represent a reservoir of D-pen which can be released by disulphide reduction in vivo, as occurs for the sym-

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metrical D-pen disulphide and the D-pen–L-cysteine mixed disulphide [7]. D-pen–protein conjugate formation may also be required for the association of D-pen with monocytes and macrophages [8,9]. Monocyte function is modified in patients treated with D-pen for rheumatoid arthritis [10,11] and it may be that binding of D-pen to cell-surface proteins mediates the action of D-pen in this condition.

The assay of D-pen–protein in human plasma requires initial separation of the protein conjugate from other forms of D-pen. Disulphides with plasma protein may form in blood and plasma samples containing D-pen, due to rapid in vitro oxidation [12] so samples need to be stabilised and separated very rapidly after collection. A reduction method is then required which will quantitatively cleave D-pen–protein disulphide releasing D-pen in a form suitable for specific assay. The intrinsic stability of disulphide bonds involving D-pen [6,13] and the nature of the subsequent assay method dictates the choice of reductant. The liberated D-pen can then be assayed using a high-performance liquid chromatographic (HPLC) assay with gold/mercury (Au/Hg) electrochemical detection [14].

EXPERIMENTAL

Specimens

Plasma samples were obtained from patients taking D-pen (250–650 mg daily) for rheumatoid arthritis, from normal, drug-free volunteers and from volunteers who had ingested a single oral dose of D-pen, 250 mg (Dista Products, Ermington, Australia). Venous blood samples (10 ml) were collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes (Vacutainer™, Becton-Dickenson, U.S.A.) and plasma was separated immediately by centrifugation (1700 *g* at 4 °C for 10 min). The protocol was approved by the St. Vincent's Hospital Ethics and Human Experimentation Committee and the Committee on Experimentation Involving Human Subjects of the University of New South Wales. These specimens were satisfactory for most aspects of assay development, but [^{14}C]D-pen–plasma albumin disulphide was also prepared in vitro so that separation and absolute recovery could be examined.

Radiolabelled D-pen–plasma albumin disulphide was prepared by the method of Yeung et al. [1]. [^{14}C]D-pen (specific activity 8.6 mCi/mmol; Amersham International, Amersham, U.K.) in non-radiolabelled D-pen carrier was incubated for 2 h at 37 °C in 10 ml of fresh heparinised plasma from a normal, drug-free volunteer in an open glass vial. A 500-μl volume of 0.5 *M* iodine in 95% (w/v) ethanol was added dropwise and the mixture was allowed to stand at room temperature for a further hour before the addition of 20 mg of solid sodium thiosulphate (to remove excess iodine). The mixture was then placed in sacs of cellulose dialysis tubing and dialysed for 48 h against disodium hydrogenphosphate buffer (5 l, 10 m*M*, pH 7.4, at 4 °C). The buffer was replaced at 24 h.

The synthetic D-pen–protein conjugate was compared with D-pen–protein conjugate formed in vivo for identity, using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Plasma samples were obtained from five Balb/c mice and four Wistar rats following parenteral administration of [^{14}C]D-

pen and each sample was separated on 11% (w/v) polyacrylamide gel, as were samples of the conjugate formed in vitro. Lanes of gel were subsequently divided into fractions and individual fractions were digested in NCS tissue solubiliser (Amersham) and counted in 12 ml of xylene surfactant-based scintillant (PCS; Amersham) by liquid scintillation spectroscopy. In each case ^{14}C activity was localised to a peak which corresponded to albumin, as identified by proximity to the 67 000 molecular weight marker. The conjugate formed in vitro was electrophoretically indistinguishable from those formed in vivo.

Separation of D-pen–protein mixed disulphide

Precipitation of protein from plasma with trichloroacetic acid (TCA), centrifugation and subsequent washing to remove low-molecular-mass compounds proved to be the most satisfactory method of separating D-pen–albumin. This method is rapid and 5%, w/v (final concentration) TCA provides quantitative protein precipitation [15]. The resultant low pH also limits thiol oxidation or disulphide exchange. The TCA precipitates were readily re-dissolved at basic pH during reduction. Plasma samples (1 ml) therefore were precipitated with TCA (BDH; 400 μl, 18%, w/v) in 100 mm × 15 mm capped polypropylene tubes (Kayline™, Bacto Labs., Liverpool, Australia), allowed to stand for 5 min at 0°C and then centrifuged (1700 *g* for 10 min at 4°) in a Bennett benchtop centrifuge. Supernatants were aspirated as completely as possible and a 10 mm × 3 mm magnetic stirring bar was placed in each tube. The precipitates were resuspended in 1 ml of 5% (w/v) TCA by stirring gently and then separated again by centrifugation at 2000 *g* for 5 min at room temperature (Hereus Christ benchtop centrifuge). The supernatants were again aspirated and discarded. This washing step was repeated. Precipitates were then air-dried prior to reduction or storage at −20°C. To check the adequacy of precipitation, [^{14}C]D-pen–protein was prepared as above. Samples (1 ml) ($n=3$) of the protein solution (mean activity 1546 dpm in 100 μl) were precipitated with 400 μl of 18% (w/v) TCA and the activity in each supernatant was counted. A mean of 27 dpm above background remained in 100 μl of supernatant after precipitation, which indicated that 97.6% of total protein-associated activity had precipitated. Radioactivity in samples was measured by liquid scintillation spectroscopy in 12 ml of PCS, using a window of 0–156 KeV, external standardisation and pre-calibrated quench correction. Samples were counted for 5 min, at which time deviation was typically under 1%.

To check the efficiency of washing, [^{14}C]D-pen–albumin disulphide was prepared as above, but the protein solutions were not dialysed. The mean initial activity in the protein solution was 11433 dpm per 100 μl ($n=2$). The samples (1 ml) were precipitated as usual and washed twice with 1 ml of 5% (w/v) TCA. The mean activity per 100 μl in the first wash was 662 dpm above background, representing 5.8% of total activity. The mean activity in the second wash was 2 dpm above background, representing 0.02% of total activity.

Reduction of D-pen–protein

Several methods of disulphide bond reduction were examined. Acid hydrolysis resulted in poor recovery. Tributylphosphine and dithiothreitol both produced

unacceptable interference with chromatography. Sodium borohydride quantitatively reduced D-pen–albumin, once conditions had been optimised. It had the additional advantage of buffering the reduction medium to pH 10.5–11.5, which enabled re-dissolution of the protein precipitate. Sodium borohydride could also be completely removed from the medium by acidification, so that it did not interfere with detection. Acidification with perchloric acid was used, since this also quantitatively re-precipitated the protein [15]. The resultant low pH limited re-oxidation of the liberated D-pen.

Reduction was carried out at room temperature in a well ventilated area (since small amounts of hydrogen evolve) and oxygen was excluded during reduction. Appreciable re-oxidation of D-pen occurred when an elevated temperature was employed or when conditions were not rigorously anoxic. Protein precipitates were reduced in the same polypropylene tubes used for precipitation and washing, to avoid losses on transfer. The magnetic stirring bar remained in each tube and a pin-hole was made in the cap to vent hydrogen during reduction. Eight precipitates were reduced simultaneously on an eight-specimen magnetic stirring block. High-purity nitrogen (CIG, Australia) was run into each tube via a PTFE catheter whilst 2 ml of 200 m*M* Tris buffer, pH 8.0 (Sigma, St. Louis, MO, U.S.A.) was dispensed from an Oxford pipettor (Oxford Labs., St. Louis, MO, U.S.A.). Smaller volumes were difficult to separate from the protein precipitate after reduction and larger volumes diluted the sample unnecessarily. The reservoir of the dispenser was bubbled with high-purity nitrogen for 1 h before use. A 100-μl volume of 250 m*M* aqueous EDTA (BDH, Epping, Australia) was added to chelate transition metals which may catalyse re-oxidation of D-pen. Octanol (50 μl; BDH) was used to limit foaming. Solid sodium borohydride (BDH) was then introduced, the gassing tube was withdrawn, the tube was capped and slow stirring was commenced. Evolution of hydrogen maintained anaerobiasis throughout reduction.

The optimal amount of sodium borohydride and the optimal duration of reduction were determined experimentally in this system, using plasma samples obtained from patients treated with D-pen. An excess of sodium borohydride is required in order to ensure complete reduction of all D-pen–albumin disulphide present. The maximum amount which can be added is limited, though, by the effect of high solute concentrations in the sample on reversed-phase separation.

Ten reaction tubes were set up, containing buffer, sample, EDTA and octanol (as above). The amounts of sodium borohydride added to the tubes were 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg. The reactions were stopped by addition of perchloric acid after 20 min and the concentrations of D-pen liberated from D-pen–albumin disulphide were measured. The amount of D-pen present after reaction rose with increasing amounts of sodium borohydride up to a maximum at between 80 and 100 mg (Fig. 1). Therefore 100 mg was chosen for subsequent use.

Reduction of D-pen–protein is not instantaneous. As time allowed for reaction lengthens, however, the opportunity for re-oxidation increases. This is because sodium borohydride becomes depleted and the resultant alkaline sodium borate

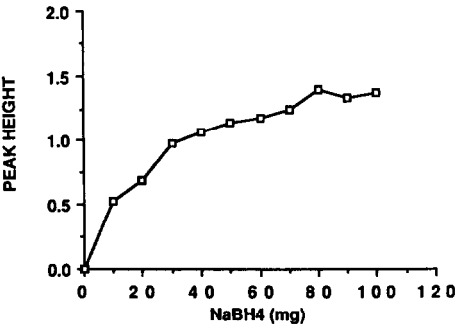

Fig. 1. D-pen release (chromatogram peak height) from human plasma samples in the presence of increasing amounts of sodium borohydride.

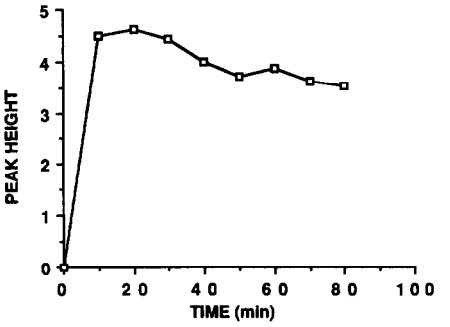

Fig. 2. Free D-pen concentration (chromatogram peak height) in human plasma samples with increasing time from the addition of sodium borohydride.

solution favours re-oxidation of D-pen. Optimal timing, therefore, is the time which will allow complete reduction without significant re-oxidation.

Nine reaction tubes were prepared, as above. Reactions were stopped at 0, 10, 20, 30, 40, 50, 60, 70 and 80 min and the D-pen concentration was measured in the supernatants. Maximal concentrations of D-pen were present between 10 and 20 min and then declined (Fig. 2). By 80 min there had been a 21% loss of D-pen from the concentration at 20 min. An incubation time of 20 min was initially adopted and later reduced to 10 min, which was found to provide quantitative reduction when other aspects of the assay had been optimised.

Terminating reduction and stabilising the specimen

Residual sodium borohydride was neutralised by the slow addition of 1 ml of ice-cold 2 *M* perchloric acid to the reaction mixture. This also provided complete protein precipitation [15] and acidification without excessive heat generation. Controlled foaming occurred, which served to deposit much of the floccular protein precipitate on the walls of the tube and permitted ready access to the medium. Floating protein (hydrogen-trapped in the precipitate presumably caused it to float) otherwise made clean removal of the medium difficult. An aliquot was pipetted into a 1.5-ml Eppendorf microcentrifuge tube and centrifuged (30 s, 8000 *g*, room temperature) in a Beckman microcentrifuge to give a water-clear

sample of approximately pH 1, which was submitted to chromatography. Acidified, separated samples showed no loss of D-pen after 18 h at −20°C. All assays were completed within 6 h after reduction.

Assay of D-pen

HPLC separation and Au/Hg electrochemical detection were used to assay the reduced D-pen [14,16]. A Varian 5020 HPLC system, including a single-piston pump and system controller, was used. The stationary phase was a 10 cm×4.6 mm Rainin "Short One" C_{18} column (3-μm particle size), maintained at room temperature. Monochloroacetic acid–sodium hydroxide buffer (BDH; 0.1 *M*, pH 3.0) with 1 g/l heptane sulphonic acid (BDH) and 6% acetonitrile (Mallinckrodt, Putney, Australia) was used as the mobile phase and all runs were isocratic at a flow-rate of 0.6 ml/min. Mobile phase was degassed by boiling and negative-pressure filtration prior to use. Rigorous removal of oxygen from mobile phase was necessary to attain maximal sensitivity and reproducibility of the assay. This was effected by boiling the mobile phase under reflux conditions and bubbling it with high-purity helium (CIG, Australia) for 3 h before commencing assay and throughout the run. Addition of a small amount of L-cysteine (BDH) to samples and standards (10 μl of 1 m*M* aqueous solution added to 200 μl of sample or standard) also assisted in maintaining the sensitivity of the assay, presumably by acting as an anti-oxidant. It eluted with the solvent front. Samples were injected using a Kortec 65A autosampler (ETP-Oxford, Ermington, Australia) equipped with either a 20- or a 50-μl injector loop.

A BAS LC-4B/19 amperometric detector with BAS TL-6A Au/Hg working electrode and a glassy carbon auxiliary electrode (Bioanalytical Systems) was linked to the column eluate line. The mercury surface of the working electrode was replaced as necessary to maintain sensitivity. The working electrode potential was maintained at +150 mV with respect to a silver/silver chloride (Ag/AgCl) electrode situated in a reservoir immediately downstream from the auxiliary and working electrodes. The detector was set at a sensitivity of 50 nA/V with a time constant of 2 s. The detector output was integrated and plotted by a Hewlett-Packard Model 3390A integrator.

Standards (0–30 μM) were prepared by adding known amounts of D-pen to reaction tubes and processing as for plasma precipitates. The equation of the calibration curve was: peak height = 0.126 [D-Pen] + 0.184 (r^2 = 0.996). A collinear standard curve was obtained using 2.1-ml (the volume in which precipitates were reduced) aliquots of D-pen standards in 0.9% (w/v) saline, acidified with 1 ml of 1 *M* perchloric acid. The slope of the calibration curve declined gradually as the electrode surface of the detector aged so standards were repeated at 3-h intervals during assay runs. The decline in detector sensitivity presumably reflected gradual loss of the mercury surface amalgam.

Summary of assay technique

Blood is collected in Venuject™ tubes containing EDTA, centrifuged immediately (1700 *g* at 4°C for 10 min) and 1 ml of plasma is transferred to a 100 mm×15 mm I.D. polypropylene tube. The plasma is precipitated with 400 μl of 18% (w/

v) TCA and after standing 5 min at 0°C, centrifuged again (1700 *g* at 4°C for 10 min). The supernatant is removed, a magnetic stirring bar is introduced and the precipitate is washed twice with 1-ml aliquots of 5% (w/v) TCA. After each addition the precipitate is stirred, centrifuged (2000 *g* at room temperature for 5 min) and the supernatant discarded. The precipitate is then air-dried at room temperature. Protein precipitates may be stored in this form. A 2-ml volume of nitrogen-saturated 200-m*M* Tris buffer at pH 8.0, 100 μl of 0.25 *M* EDTA and 50 μl of octanol are then added. Oxygen is displaced from the buffer by bubbling with high-purity nitrogen and nitrogen blanketting of the dispenser reservoir is maintained throughout. Nitrogen is run into the tubes through PTFE lines until sodium borohydride (100 mg, solid) is added and slow stirring is started. The nitrogen tube is then withdrawn and the tube is capped. A pin-hole is made in the cap prior to addition of the sodium borohydride. The reaction proceeds at room temperature for 10 min with stirring. The tube is then transferred to an ice bath and 1 ml of ice-cold 2 *M* perchloric acid is added dropwise. The tube is briefly stirred and an aliquot of the supernatant is drawn by pipette from below the floating layer of flocculated protein. This is placed in a 1.5-ml Eppendorf tube and centrifuged in a Beckman microcentrifuge (8000 *g* at room temperature for 30 s). The supernatant is decanted, 10 μl of 1 m*M* L-cysteine are added to 200 μl and this is injected by autosampler onto the HPLC instrument for chromatography.

RESULTS AND DISCUSSION

Recovery of D-pen released during chemical reduction was examined using [^{14}C]D-pen–protein, which was prepared as above. The mean activity in 100 μl of the protein solution ($n=3$) was 1546 dpm. A mean of 2.4% remained in solution after precipitation of 1 ml with 400 μl of 18% (w/v) TCA. The precipitate was reduced, as above, and a mean of 92.7% of the precipitated activity was recovered in the supernatant. The reason for the deficit was not elucidated, but could have been due to incomplete reduction of disulphides, the presence of small amounts of chemically distinct, irreducible D-pen–protein conjugate or because some of the liberated D-pen reformed disulphides in the reduction medium. Internal standardisation of the reduction phase of the assay, using another thiol–protein disulphide may have corrected for incomplete recovery. However, no suitable compound was identified. Recovery was complete in subsequent phases of the assay.

Following chemical reduction and HPLC separation, D-pen eluted at 4.66 min as a discrete peak on the tail of the solvent front. No interference was present in this area of chromatograms of blank samples. A typical chromatogram (volunteer, after a single 250-mg dose of D-pen) is shown in Fig. 3. The reproducibility of the assay was examined using pooled patient plasma. The intra-assay coefficient of variation was 4.6% at 23.9 μ*M* ($n=7$) and 2.9% ($n=6$) at a lower concentration (peak height measured; not compared with standards). The sensitivity was 1.2 μ*M* of D-pen–protein in plasma (2.9 ng on-column, using a 50-μl injection loop) with a signal-to-noise ratio greater than 2.

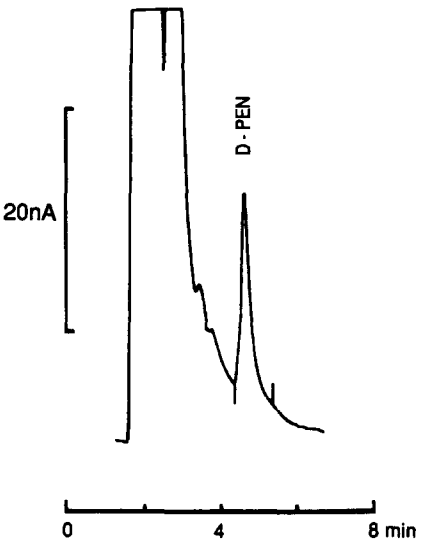

Fig. 3. Chromatogram of D-pen, released from D-pen–plasma protein conjugate (9.3 μM), from a human volunteer after a 250-mg oral dose of D-pen. Retention time is 4.66 min.

Assays for other protein-bound thiols, including glutathione [17,18], homocysteine [19] and captopril [20], have been published, but some reports have not included information on recovery or reproducibility. In each case, liberated thiol is either assayed indirectly or is derivatised for HPLC detection. The methodology developed here for D-pen is also likely to be applicable to protein disulphides of these and other thiols. The virtues of specificity, sensitivity and ability to measure directly the underivatised thiol may favour it above current assays for these protein conjugates.

The D-pen–albumin disulphide assay is currently being used to study the oxidation of D-pen to albumin disulphide in human volunteers and patients with rheumatoid arthritis. It is anticipated that this work will explain certain paradoxes in human D-pen pharmacokinetics, such as urinary elimination of D-pen metabolites up to three months after ceasing therapy [21,22]. The work will also provide information on the potential importance of D-pen-protein conjugates in the mode of action of D-pen [8,9].

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