BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **14**: 535–540 (2000)

ORIGINAL RESEARCH

High performance liquid chromatography analysis of D-penicillamine by derivatization with N-(1-pyrenyl) maleimide (NPM)

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Received 10 December 1999; revised 2 November 2000; accepted 2 February 2000

ABSTRACT: D-Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid), a well-known heavy metal chelator, is the drug of choice in the treatment of Wilson's disease and is also effective for the treatment of several disorders including rheumatoid arthritis, primary biliary cirrhosis, scleroderma, fibrotic lung diseases and progressive systemic sclerosis. The method proposed incorporates a technique, previously developed in our laboratory, that utilizes the derivatizing agent N-(1-pyrenyl)maleimide (NPM) and reversed-phase high-performance liquid chromatography (HPLC). The coefficients of variation for within-run precision and between-run precision for 500 nM standard D-penicillamine (D-pen) were 2.27% and 2.23%, respectively. Female Sprague–Dawley rats were given 1 g/kg D-pen i.p. and the amounts of D-pen in liver, kidney, brain and plasma were subsequently analyzed. This assay is rapid, sensitive and reproducible for determining D-pen in biological samples. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION

D-Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) is the drug of choice in the treatment of Wilson's disease due to its amino thiol properties and chelating abilities. D-Penicillamine (D-pen) is also useful in the treatment of heavy metal poisoning, rheumatoid arthritis, primary biliary cirrhosis, cystinuria, scleroderma and progressive systemic sclerosis (Chapela *et al.*, 1986; Davis, 1984; Levy *et al.*, 1983; Kang *et al.*, 1982; Roelofs *et al.*, 1979). Despite its wide use in medicine, the pharmacokinetics of D-pen in man is not well established, partly due to the lack of a suitable detection method for D-pen (Crawhall *et al.*, 1979 and Wolf-Huess, 1987).

After prolonged treatment, D-pen induces undesirable toxic effects (Jaffe, 1986; Kean *et al.*, 1984; Menara *et al.*, 1992). The most undesirable effects include hypersensitivity, nephrotic syndrome, myasthenia gravis and increased excretion of essential elements (Brewer, 1995; Van Caillie-Bertrand *et al.*, 1985). Therefore, it is necessary to detect tissue, serum and/or urine levels of D-pen in order to assess the degree of possible toxicity. The quantitation of D-pen in biological samples is complicated by the occurrence of many different forms; free thiol; internal disulfide; mixed disulfide with cysteine; metabolite S-methyl-D-pen; and D-pen bound to plasma proteins (Muijsers *et al.*, 1979). Previous methods of detecting these multiple forms of D-pen include cation-exchange chromatography (Muijsers *et al.*, 1979), radioimmunoassay (Assem and Vickers, 1974), high performance liquid chromatography (Hidayat *et al.*, 1997; Kucharczyk and Shahinian, 1981; Lankmayr *et al.*, 1981; Miners *et al.*, 1983; Nakashima *et al.*, 1989; Saetre and Rabenstein, 1978), and gas chromatography (Kucharczyk and Shahinian, 1981). Sensitivity, reproducibility, time-consuming procedures and cumbersome manipulation with multiple samples are all inherent limitations of these assay methods (Miners *et al.*, 1983). Due to these inherent problems, no quantitative data are available on the relative proportion of D-pen metabolites in different disease states.

The assay method proposed incorporates a technique previously developed in our laboratory utilizing the derivatizing agent N-(1-pyrenyl)maleimide (NPM; Winters *et al.*, 1995). NPM forms flourescent derivatives with compounds containing a free sulfhydryl group. The present method uses NPM to form a flourescent derivative with D-pen according to the reaction shown in Fig. 1. We have utilized this derivatization technique and reverse-phase HPLC to measure D-pen in liver, kidney, brain and plasma samples.

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Abbreviations used: CV, coefficient of variation; CYS, cysteine; DBPM, N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide; D-pen, D-penicillamine; GSH, glutathione; HPLC, high performance liquid chromatography; N/A, not applicable; NPM, N-(1-pyrenly) maleimide; SD, standard deviation; S/N, signal-to-noise ratio.

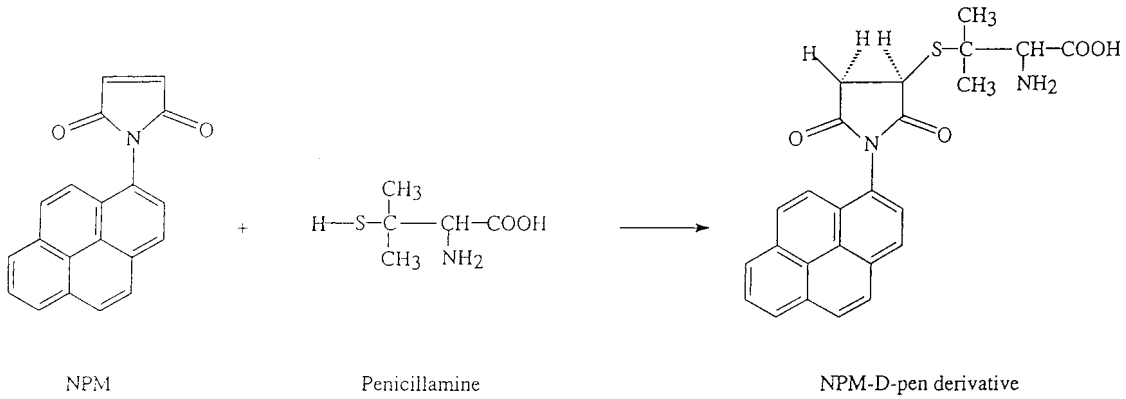

Figure 1. Formation of fluorescent NPM–D-pen adduct.

EXPERIMENTAL

Reagents and chemicals

Acetonitrile, acetic acid, phosphoric acid and Tris–EDTA buffer (all HPLC grade) were purchased from Fisher (St Louis, MO, USA). NPM was obtained from Aldrich (Milwaukee, WI, USA). D-Penicillamine was purchased from Sigma (St Louis, MO, USA).

Animals

Adult male Sprague–Dawley rats weighing 100–150 g were obtained from Charles River Laboratories. The rats were kept in stainless steel cages in a temperature controlled (25°C) room equipped to maintain a 12 h light–dark cycle. Standard rat chow (Purina rat chow) and water were given *ad libitum*. D-Pen was given intraperitonealy 100 mg/kg body weight in 1 mL saline. Blood was collected via intra-cardiac puncture 30 min after a dosage of D-Pen, followed by subsequent removal of liver, kidney and brain tissues. Samples were homogenized (Tissue Tearor, model 985-370) on ice in Tris–EDTA buffer for 2 min in 5 s alternate intervals. All samples were immediately assayed.

HPLC system

The HPLC system (Shimadzu) comprised a model LC10A pump, a Rheodyne injection valve with a 20 μL loop and a model RF 535 fluorometer operating at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The HPLC column (Astec, Whippany, NJ, USA) was 100 × 4.6 mm i.d. and contained 3 μm particles of C_{18} packing material. Quantitation of the peaks was performed with a Chromatopac, Model CR601 integrator (Shimadzu). The mobile phase was 40% acetonitrile, 60% water, 1 mL/L *o*-phosphoric acid, and 1 mL/L acetic acid. The NPM derivatives were eluted from the column at a flow rate of 0.50 mL/min.

Assay procedures

Calibration and relative recovery. Calibration curves of D-pen were constructed by injecting 20 μL of NPM-derivatized standards containing 250 mg of tissue and standards in no tissue matrix. Linearity in samples was obtained over the full range 4–2500 nM (r = 0.997), while linearity in standards was obtained over the full range of 4–2500 nM (r = 0.999). Relative recovery was determined by spiking tissue samples with various concentrations of D-pen and comparing the results to those obtained from aqueous samples supplemented with the same concentrations (Table 1).

Sample derivatization. Plasma and homogenates of liver, kidney and brain tissues from Sprague–Dawley rats were derivatized with NPM. A Tris–EDTA buffer (240 μL) and 1 mM NPM solution (750 μL) was added to diluted samples (10 μL). The resulting solution was vortexed and incubated at room temperature for 30 min before acidification with 5 μL of 1/6 M HCl to stop the reaction. There were no significant differences in D-pen derivative peak areas when a sample was incubated from 30 to 45 min (Fig. 2). It should be noted that the reaction is complete at 30 min, but must be stabilized with the addition of HCl within 45 min. Filtration through a 0.2 μm acrodisc was performed and the derivatized samples were injected onto a 3 μm C_{18} column in a reverse-phase HPLC system. The NPM–penicillamine derivatives remained stable for at least 2 weeks when stored at 4°C with a deviation in peak areas less than 2% in all sample matrices.

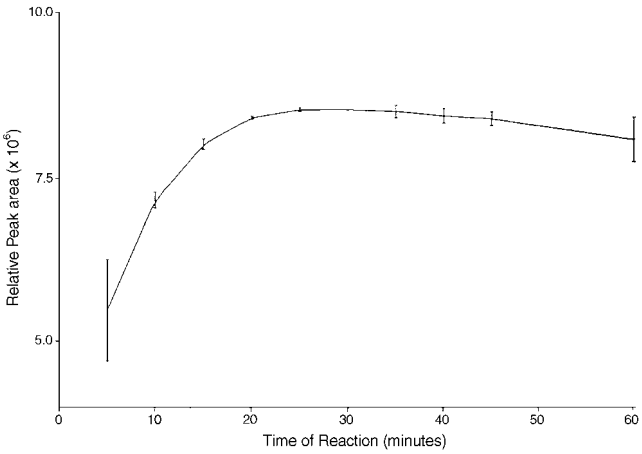

Figure 2. Reaction time of NPM–D-pen adduct (n = 3). Peak areas are reported as mean values ± standard deviation (SD).

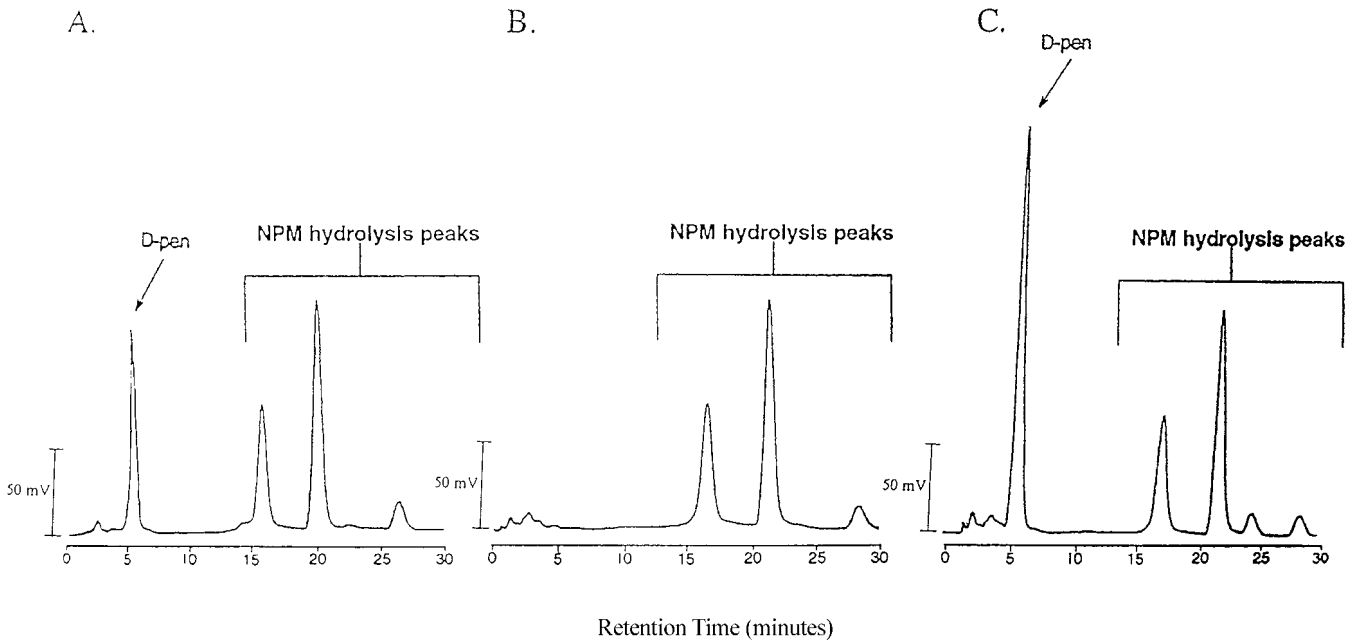

Figure 3. (A) Standard chromatogram containing peaks from the NPM–D-pen (500 nM) adduct. (B) Chromatogram obtained from plasma sample (no D-pen peak). (C) Chromatogram showing D-pen peak from a plasma sample. The attenuation for all chromatograms is 256 mV/full scale.

Protein determination

The Bradford method was used to determine the protein content of the cell samples (Bradford, 1976). Concentrated Coomassie Blue (Bio-Rad) was diluted 1:4 (v/v) with distilled water. A total of 2.5 mL of the diluted reagent were added to 0.05 mL of a bovine serum albumin standard solution which contained 10–100 μg from a 1 mg/mL stock solution. The mixture was incubated at 37°C for 5 min and the absorbance was measured at 595 nm. The samples were subjected to appropriate dilutions and 0.05 mL of each sample was used for the protein assay.

RESULTS

The NPM–D-pen adducts produced by *in situ* derivatization of biological samples were rapidly separated by the HPLC system described previously. The chromatogram pictured in Fig. 3(A) confirms the separation of the NPM–penicillamine adduct from the NPM hydrolysis peaks. Chromatograms prepared from the biological samples [Fig. 3(B) and 3(C)] illustrate the clearly defined separation of the NPM–D-pen adduct from the NPM hydrolysis peaks and the lack of NPM-glutathione or NPM-cysteine peaks. In order to further illustrate the lack of interference from NPM–glutathione and NPM–cysteine peaks, chromatograms of samples containing a mixture of all three thiols (500 nM each) were obtained and are shown in Fig. 4. Chromatograms of plasma before and after spiking with 125 nM D-pen confirm the location and presence of the NPM-D-pen peak (Fig. 5). The retention time for the NPM–penicillamine adduct was 5.6 min.

Calibration curve

A calibration curve was constructed by plotting integrated peak areas vs D-pen concentrations at regular intervals. Linearity was displayed for D-pen concentrations ranging from 4 to 2500 nM ($r = 0.999$). In addition, linearity in all sample matrices was displayed for the same range ($r = 0.997$).

Sensitivity, stability, reproducibility and relative recovery

The lower limit of detection for D-pen utilizing this method was established at 4 nM (S/N = 3). Tissue and plasma samples derivatized with NPM and maintained at 4°C remained stable for at least 2 weeks with less than 2% deviation in all sample matrices. The coefficients of variation (CV) for within-run precision and between-run precision in standards and samples are reported in Table 1. Relative recovery experiments were performed by spiking tissue samples with known concentrations of D-pen and comparing the results to chromatograms from a standard curve using the same D-pen concentration (Fig. 5). The mean relative recovery of five separate experiments is reported in Table 1.

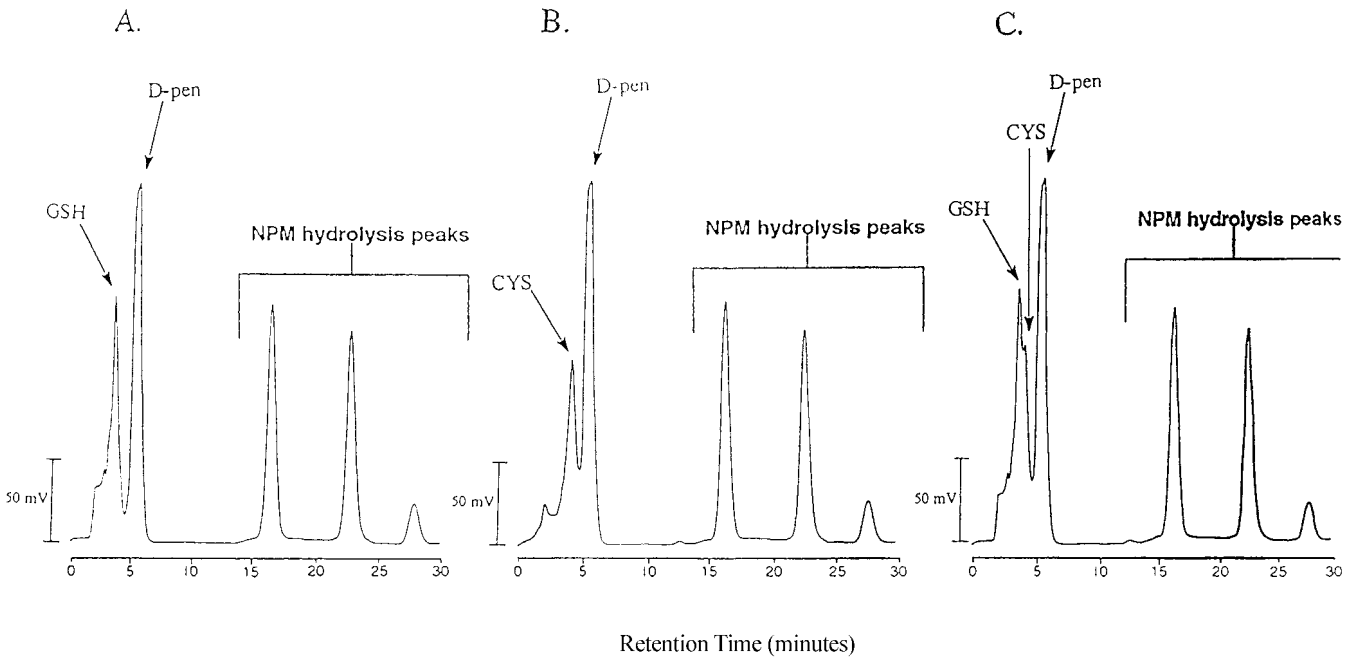

Figure 4. (A) Chromatogram of a mixture of GSH (500 nM) and D-pen (500 nM). (B) Chromatogram of a mixture of CYS (500 nM) and D-pen (500 nM). (C) Chromatogram of a mixture of GSH (500 nM), CYS (500 nM) and D-pen (500 nM). The attenuation for all chromatograms is 256 mV/full scale.

Results of biological samples

The concentration of D-pen in samples of plasma, kidney, liver and brain tissues taken from Sprague–Dawley rats is shown in Table 2. The rats were sacrificed and the samples were obtained 30 min after receiving intraperitoneal injections of 100 mg/kg D-pen. The concentration of D-pen in kidney samples was higher than in plasma or other tissue homogenates. According to the data, D-pen accumulation following a 30-min exposure to 100 mg/kg is greatest in kidney tissue and lowest in brain tissue.

DISCUSSION

Since the discovery of the therapeutic role of D-pen in the treatment of Wilson's disease (Walshe, 1956), numerous

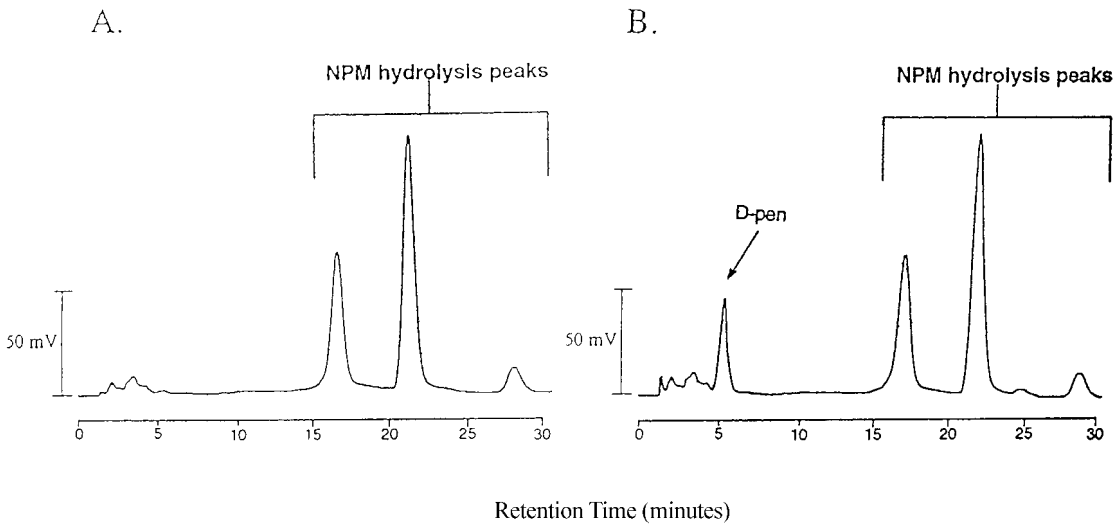

Figure 5. (A) Chromatogram of plasma before spiking with D-pen. (B) Chromatogram of plasma spiked with 125 nM D-pen. The attenuation for all chromatograms is 256 mV/full scale.

Table 1. D-Pen (1250 nM) in sample matrices (n = 9) and standards (n = 9) for within-run and between-run precision. Relative recovery is reported as the average relative recovery (± standard deviation) of five samples spiked with 125–2500 nM D-pen in each sample matrix. N/A = not applicable

Sample matrix	Kidney	Brain	Liver	Plasma	Standard
Between-run precision (n = 9)	3.57%	4.35%	4.46%	4.57%	2.23%
Within-run precision (n = 9)	3.86%	1.81%	0.48%	3.21%	2.27%
Relative recovery (n = 5)	105.4% ± 3.2%	104.2% ± 6.3%	97.2% ± 3.1%	115.1% ± 13.4%	N/A

other possible treatments have been identified; however, D-pen remains the drug of choice. The racemic D,L-form of penicillamine was initially used, but animal studies have since identified the D-form as less toxic (Walshe, 1968). Despite the beneficial effects of D-pen, a degree of toxicity is related to characteristic concentrations of accumulated D-pen and constant monitoring of plasma and urine D-pen levels of patients is required (Levy *et al.*, 1983; Wolf-Huess, 1987). Consequently, a rapid, sensitive, reproducible assay for the determination of D-pen in biological samples is desired.

Assay methods utilizing cation-exchange chromatography, radioimmunoassay, HPLC and gas chromatography have been identified, each with its own limitations (Wolf-Huess, 1987). The cation-exchange chromatographic method described by Muijsers *et al.* (1979) is useful in determining of all forms of D-pen; however, extensive manipulation of samples makes it undesirable for clinical use. Assem's radioimmunoassay is not developed sufficiently for clinical use (Assem and Vickers, 1974). The HPLC method outline by Saetre and Rabenstein (1978) requires the formation of a custom-made mercury-based electrochemical detector, while that described by Hidayat *et al.* (1997) requires the formation of a tungsten-based electrochemical detector. Nakashima *et al.* (1989) has provided a method for the determination of D-pen using N-[4-(6-dimethylamino-2-benzofuranyl)-phenyl]maleimide (DBPM) in clinical settings; however, this method requires specific reaction conditions (60°C) and the samples are only stable for 24 h after derivatization. In addition, synthesis of the derivatizing agent is not practical in the clinical setting. The thiol determination method described by Nakashima *et al.* (1989) utilizing the same derivatizing agent, DBPM, did not include D-pen among the thiols analyzed in biological samples, making it difficult to determine the fate of D-pen detection in biological samples. The fluorometric HPLC method utilizing 5-dimethylaminonaphthalene-1-sulfonylaziridine and N-[*p*-(2-benzoxazolyl)phenyl]maleimide as derivatizing agents requires lengthy incubation periods, overnight at 37°C (Lankmayr *et al.*, 1981; Miners *et al.*, 1983). The gas chromatographic assays are not only laborious and time consuming (requiring 2 working days), but they are also not sensitive enough to detect the presence of low levels of D-pen in man (Kucharczyk and Shahininan, 1981; Wolf-Huess, 1987). In contrast, the NPM method was shown to be reproducible, sensitive, specific and easy to use, with accurate recovery of D-pen in biological samples.

The D-pen method described in the present study is useful to the extent that it can be run under ambient conditions and requires about 40 min to run after sample preparation. Moreover, the analysis, being an HPLC method, is adaptable for automation of the assay. The linearity of the assay ranges from 4 to 2500 nM and has low coefficients of variation for reproducibility. Furthermore, application of the present assay to biological materials, including plasma, liver, kidney and brain samples has been demonstrated by the accurate relative recovery of D-pen. Finally, the implementation of this method for determining D-pen levels in human tissue and plasma in a clinical setting is possible.

Table 2. D-Pen in tissues 30 min after intraperitoneal injection of 100 mg/kg D-pen. After derivatization with NPM, the NPM–D-pen adducts formed were measured by HPLC and sample D-pen concentration were compared to known standards

	D-pen concentration	
Sample	Mean ± SD (n = 3)	Control
Plasma	6.46 ± 0.37 μmol/L	ND
Liver	0.41 ± 0.12 nmol/mg protein	ND
Kidney	0.55 ± 0.04 nmol/mg protein	ND
Brain	0.15 ± 0.02 nmol/mg protein	ND

REFERENCES

Assem ESK, Vickers MR. 1974. Immunological response to penicillamine in penicillin-allergic patients and in normal subjects. *Postgraduate Medical Journal* **50**:10.

Brewer GJ. 1995. Practical recommendations and new therapies for Wilson's disease. *Drugs* **50**:240.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**:248.

Chapela R, Zuniga G, Selman M. 1986. D-penicillamine in the therapy of fibrotic lung diseases. *International Journal of Clinical Pharmacology, therapy and toxicology* **24**:16.

Crawhall JC, Lecavalier D, Ryan P. 1979. Penicillamine, its metabolism and therapeutic applications: a review. *Biopharmacy and Drug Disposal* **1**:73.

Davis P. 1984. Penicillamine metal chelates and their possible importance in rheumatoid arthritis—a brief review. *Clinical Investigative Medicine* **7**:41.

Hidayat A, Hibbert DB, Alexander PW. 1997. Amperometric detection of organic thiols at a tungsten wire electrode following their separation by liquid chromatography. *Journal of Chromatography, B*, **693**:139.

Jaffe IA. 1986. Adverse effects profile of sulfhydryl compounds in man. *American Journal of Medicine* **80**:471.

Kang B, Veres-Thorner C, Heredia R, Cha E, Bose S, Schwartz M. 1982. Successful treatment of far-advanced progressive systemic sclerosis by D-penicillamine. *Journal of Allergy and Clinical Immunology* **69**:297.

Kean WF, Bellamy N, Lock CJL, Adachi J. 1984. Bio-inorganic effects of D-penicillamine in children and in the elderly. *Clinical Investigative Medicine* **7**:77.

Kucharczyk N, Shahinian S. 1981. An overview of assay methods for D-penicillamine. *Journal of Rheumatology* (Suppl) **7**:28.

Lankmayr EP, Budna KW, Muller K. 1981. D-Penicillamine in serum by fluorescence derivatization and liquid column chromatography. *Journal of Chromatography* **222**:249.

Levy RS, Fisher M, Alter JN. 1983. Penicillamine: review and cutaneous manifestations. *Journal of the American Academy of Dermatology* **8**:548.

Menara M, Zancan L, Sturniolo GC. 1992. Penicillamine hepatotoxicity in the treatment of Wilson's disease. *Journal of Pediatric Gastroenterology and Nutrition* **14**:353.

Miners, Fearnley I, Smith KJ, Birkett DJ. 1983. D-penicillamine in plasma by fluorescence derivatization with N-[p-(2-benzoxazolyl)-phenyl]maleimide and high performance liquid chromatography. *Journal of Chromatograply B* **275**:89.

Muijsers AO, Van de Stadt RJ, Henrichs AMA, Van der Korst JK. 1979. Determination of D-penicillamine in serum and urine of patients with rheumatoid arthritis. *Clinica Chimica Acta* **94**:173.

Nakashima K, Umekawa C, Nakatsuji S, Akiyama S. 1989. High-performance liquid chromatography/chemiluminescence determination of biological thiols with N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide. *Biomedical Chromatography* **3**:39.

Roelofs RI, Saavedra De Arango G, Law PK, Kinsman D, Buchanan DC. 1979. Treatment of Duchenne's muscular dystrophy with penicillamine. Results of a double-blind trial. *Archives in Neurology* **36**:266.

Saetre R, Rabenstein DL. 1978. Determination of penicillamine in blood and urine in high-performance liquid chromatography. *Analytical Chemistry* **50**:276.

Van Caillie-Bertrand M, Degenhart HJ, Luijendijk I, Bouquet J, Sinaasappel M. 1985. Wilson's disease: assessment of D-penicillamine treatment. *Archives of Disease in Childhood* **60**:652.

Walshe JM. 1956. Penicillamine: a new oral therapy for Wilson's disease. *American Journal of Medicine* **21**:487.

Walshe JM. 1968. Toxic reactions to penicillamine in patients with Wilson's disease. *Postgraduate Medical Journal* **44**:(Suppl): 6.

Winters R, Zukowski J, Ercal N, Matthews RH, Spitz DR. 1995. Analysis of glutathione, glutathione disulfide, cysteine, homocysteine, and other biological thiols by high-performance liquid chromatography following derivatization by N-(1-pyrenyl)maleimide. *Analytical Biochemistry* **227**:14.

Wolf-Heuss EM. 1987. Penicillamine. *Methods in Enzymology, Separation Analysis* **143**:186.

