Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 803 (2004) 299–304
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Plasma D-penicillamine redox state evaluation by capillary electrophoresis with laser-induced fluorescence

Angelo Zinellu, Ciriaco Carru*, Salvatore Sotgia, Luca Deiana

Chair of Clinical Biochemistry, University of Sassari, Viale San Pietro 43/B, I-07100 Sassari, Italy

Received 23 October 2003; received in revised form 5 January 2004; accepted 7 January 2004

Abstract

D-Penicillamine (D-Pen) is a thiol drug used in the treatment of Wilson's disease, rheumatoid arthritis, metal intoxication and cystinuria. We have recently described a new capillary electrophoresis (CE) method to measure physiological thiols, in which separation of total plasma homocysteine, cysteine, cysteinylglycine, glutathione is achieved using the organic base *N*-methyl-D-glucamine in the run buffer. In this paper, we present an improvement of our method that allows a baseline separation of total plasma D-Pen from the physiological thiols. Moreover, reduced, free and protein-bound forms of drug are measured by varying the order of disulfide reduction with tributylphosphine and proteins precipitation with 5-sulphosalicylic acid (SSA). After derivatization with 5-iodoacetamidofluorescein (5-IAF), samples are separated and measured by capillary electrophoresis with laser-induced fluorescence in an uncoated fused-silica capillary (57 cm × 75 μm i.d.) using a phosphate/borate run buffer pH 11.4. In these conditions, the migration time of D-Pen is about 7 min and the time required for each analysis is roughly 10 min. The proposed method has been utilized to measure the various forms of the drug in a D-Pen administered Wilson's disease patient.

Keywords: D-Penicillamine; Thiols

1. Introduction

Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) is an unphysiological sulfur-containing amino acid that belongs to the aminothiols family. This compound is derived from hydrolytic degradation of penicillin [1–3] but it does not have antibiotic activity. It differs from the cysteine only for the presence of two methyl groups on the ß-carbon and it exists in D- and L-enantiomeric forms that show different biological and toxicological properties. D-Pen (*S*-isomer) is a highly potent therapeutic agent used for many years in the treatment of various illnesses. It is the drug of first choice for patients with Wilson's disease [4], an autosomal recessive disorder of copper transport [5]. It is able to enhance the urinary excretion of others heavy metals such as lead, arsenic, mercury and zinc and therefore, is used as an oral chelating agent to treat conventional heavy metals intoxication [6]. It is also used as antifibrotic agent to treat scleroderma [7] and as antirheumatic drug to treat patients with active rheumatoid arthritis [8]. D-Pen reduces excess of cystine excretion in cystinuria, another rare inherited disease affecting the active transport of the di-amino acids cystine, ornithine, lysine and arginine across the renal tubule and the small intestine [9]. Mechanisms of action of D-Pen in mentioned diseases are various. It is a reductive chelating agent [10], it appears to inhibit collagen deposition by interfering with the intramolecular cross-linking of mature collagen [11], it has immunomodulatory-anti-inflammatory properties [12–14] and it is able to make dimers through disulfide interchange or direct reaction with other physiological thiols [15,16]. For example, in cystinuria, D-Pen-cysteine disulfide formation, which is a dimer more soluble than cystine, prevents the clinical manifestation of the pathology [16]. This suggests that it may also represent a potential drug for the hyperhomocyst(e)inemia and hypercyst(e)inemia treatment, two independent risk factors for atherosclerosis and cardiovascular disease [17,18]. According to us, it is not

Abbreviations: CE-LIF, laser-induced fluorescence capillary electrophoresis; D-Pen, D-penicillamine; 5-IAF, 5-iodoacetamidofluorescein; SSA, 5-sulphosalicylic acid; TBP, tri-*n*-butylphosphine

* Corresponding author. Fax: +39-079-228120.
E-mail addresses: angelozinellu@libero.it (A. Zinellu), carru@uniss.it (C. Carru).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.01.002

sufficient to measure only one form or another of D-Pen (i.e., total or free) in order to evaluate the extent of disulphide interchange reactions and its therapeutic effectiveness and to accomplish more accurate pharmacokinetic studies. Exhaustive information may be obtained only by discriminating all the forms of D-Pen. However it is complicated by the presence of physiological thiols such as cysteine, cysteinylglycine, homocysteine and glutathione, that interfere when conventional methods with SH-specific reagents were used. Measurement of D-Pen in plasma can be done by colorimetric method [19–21], radioimmunoassay [22,23], anion-exchange chromatography amino acids auto-analyzer [24], high-performance liquid chromatography with UV [25], fluorescence [26,27] and electrochemical detection [28–30], gas chromatography [31] and high-performance capillary electrophoresis (CE) [32]. None of them are sensitive and selective enough to provide a suitable assay for the discrimination of different drug forms. We have recently described a new capillary electrophoresis method with laser-induced fluorescence detection to quantify total plasma thiols [33]. In the present paper, we report a modification of this assay that allows, for the first time, the measurement of reduced, free, oxidized, protein bound and total D-Pen by laser-induced fluorescence capillary electrophoresis. The performance of the analytical method was assessed by measuring the various forms of the drug in the plasma sample taken from a subject affected by Wilson's disease and administered with D-Penicillamine.

2. Experimental

2.1. Chemicals

Pharmaceutical preparation of D-penicillamine (Pemine®) from Ely Lilly Italia (Sesto Fiorentino (FI), Italia) was obtained. Reduced D-penicillamine, homocystine, homocysteine, cystine, cysteine, oxidized cysteinylglycine, reduced cysteinylglycine, oxidized glutathione, reduced glutathione, glutamylcysteine, Na_3PO_4, H_3BO_3, *N*-methyl-D-glucamine, *N*,*N*-dymethilphormamide (DMF), tri-*n*-butylphosphine (TBP), NaOH, 5-iodoacetamidofluorescein (5-IAF), and 5-sulphosalicylic acid (SSA) were obtained from Sigma (St. Louis, USA). Membrane filters (0.45 μm), obtained from Millipore (Bedford, USA), were used to filter all buffer solution before CE analysis. The water used for the experiments was Milli-Q grade.

2.2. Sample collection and preparation

Blood from a Wilson's disease patient treated with an oral dose of 1.2 g/day of Pemine® was collected by venipuncture into evacuated tubes containing EDTA, and immediately centrifuged at 3000 × *g* at 4 °C for 2 min. Plasma was aliquoted and then stored at −80 °C, analyses were performed within 1 week.

2.3. Total plasma thiols determination (procedure 1)

Two hundred microlitres of plasma sample were mixed with 20 μl of 10% (v/v) TBP in DMF for 10 min to reduce disulfide bounds. Plasma proteins were then precipitated by adding 200 μl of 5-sulphosalicylic acid (6%) and removed by centrifugation (2000 × *g* at 4 °C for 5 min). Supernatant (100 μl) was derivatized by adding 100 μl of 300 mmol/l sodium phosphate buffer pH 12.5 and 25 μl of 4 mmol/l of 5-IAF. After 15 min at room temperature, derivatized samples were diluted 100-fold in water and analyzed by capillary electrophoresis.

2.4. Protein-bound thiols determination (procedure 2)

Two hundred microlitres of plasma sample were deproteinized by adding 50 μl of SSA (15%) and centrifuged at 2000 × *g* at 4 °C for 5 min. Following protein precipitation, the supernatant was discarded and protein pellet was dissolved in 200 μl of Tris buffer (0.5 mol/l pH 8.5). Twenty microlitres of 10% (v/v) TBP in DMF were added for 10 min to release protein-bound thiols. Plasma proteins were then precipitated by adding 200 μl of SSA (6%) and removed by centrifugation (2000 × *g* at 4 °C for 5 min). One hundred microlitres of supernatant were mixed with 100 μl of 300 mmol/l sodium phosphate buffer pH 12.5 and 25 μl of 4 mmol/l of 5-IAF. After 15 min at room temperature, derivatized samples were diluted 100-fold in water and analyzed by capillary electrophoresis.

2.5. Free plasma thiols determination (procedure 3)

Two hundred microlitres of plasma sample were deproteinized by adding 50 μl of SSA (15%) and centrifuged at 2000 × *g* at 4 °C for 5 min. To 100 μl of supernatant were added 100 μl of 0.5 mmol/l of NaOH and 20 μl of 10% (v/v) TBP in DMF for 10 min. Afterwards, 50 μl of sample were mixed with 100 μl of 150 mmol/l sodium phosphate buffer pH 12.5 and 15 μl of 0.8 mmol/l of 5-IAF. After 15 min at room temperature, derivatized samples were diluted 100-fold in water and analyzed by capillary electrophoresis.

2.6. Reduced plasma thiols determination (procedure 4)

Two hundred microlitres of plasma sample were deproteinized by adding 50 μl of SSA (15%) and centrifuged at 2000 × *g* at 4 °C for 5 min. To 150 μl of supernatant were added 30 μl of 1 mmol/l of NaOH. Afterwards, 50 μl of sample were mixed with 100 μl of sodium phosphate buffer (100 mmol/l) pH 12.5 and 15 μl of 0.8 mmol/l of 5-IAF. After 15 min at room temperature, derivatized samples were diluted 100-fold in water and analyzed by capillary electrophoresis.

Oxidized penicillamine (disulfide) were calculated by difference (free minus reduced form).

2.7. Capillary electrophoresis

The P/ACE 5510 capillary electrophoresis system equipped with laser-induced fluorescence was used (Beckman instruments, CA, USA). The P/ACE 5510 system was fitted with a 30 kV power supply with a current limit of 250 μA. The dimension of the uncoated fused-silica capillary was 75 μm i.d. and 57 cm length (50 cm to the detection window). Analysis was performed applying 14 nl of sample under nitrogen pressure (0.5 psi) for 2 s using a 18 mmol/l sodium phosphate, 14.5 mmol/l boric acid as electrolyte solution with 75 mmol/l *N*-methyl-D-glucamine, pH 11.4. The separating conditions (22 kV, 150 μA at normal polarity) were reached in 20 s and held at a constant voltage for 9 min. All separations were carried out at 40 °C. After each run, no rinse was necessary; capillary was equilibrated with 1 min of run buffer.

3. Results and discussion

3.1. Capillary electrophoresis optimization

We have recently described a new rapid CE-LIF method for the measurement of total plasma thiols in which 5-IAF was used as thiol specific fluorogenic reagent [33]. We utilized the same fluorophore to derivatize also the D-Pen thiol drug. 5-IAF form a fluorescent derivative with D-Pen according to the reaction described in Fig. 1. To evaluate the times of derivatization 100 mmol/l of D-Pen were mixed with 4 mmol/l of 5-IAF in a 300 mmol/l sodium phosphate buffer, pH 12.5 and incubated for 120 min at room temperature. In these conditions the reaction reached the plateau in 10 min (data not shown), similarly to derivatization times of physiological thiols described in our previous paper. The measurement of the reduced, oxidized, free, protein-bound and total forms of D-Pen was obtained by modifying the derivatization reaction and the run buffer conditions described in our previous method used for total plasma thiols analysis [33]. Particularly, reduced and free D-Pen forms were measured by varying the order of TBP disulfide reduction and SSA proteins precipitation, while derivatization reaction was carried out using 0.8 mmol/l of 5-IAF concentration instead of 4 mmol/l, employed for total and protein-bound forms. The 5-IAF concentration decrease reduces some interfering reagent peaks to acceptable levels. In the previous work we used a 5 mmol/l sodium phosphate, 4 mmol/l boric acid, 75 mmol/l *N*-methyl-D-glucamine, at pH 11 as run buffer to obtain a baseline separation of physiological thiols. However, in these conditions, D-Pen and homocysteine peaks were unresolved, as seen in Fig. 2a, so that it was necessary to extend the run time separation to obtain a satisfactory resolution. Since the property of *N*-methyl-D-glucamine is to mask the silanol groups on the uncoated capillary surface causing a decrease of EOF, longer separation times could be obtained by incrementing its concentration on the run buffer. But as described in our previous paper, a concentration of this compound over 75 mmol/l results in a peak broadening and in a loss of

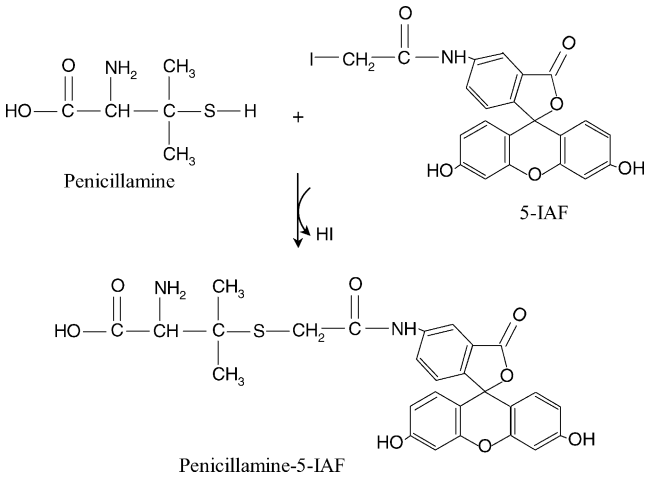

Fig. 1. Hypothetical conjugation reaction between 5-IAF and D-Pen.

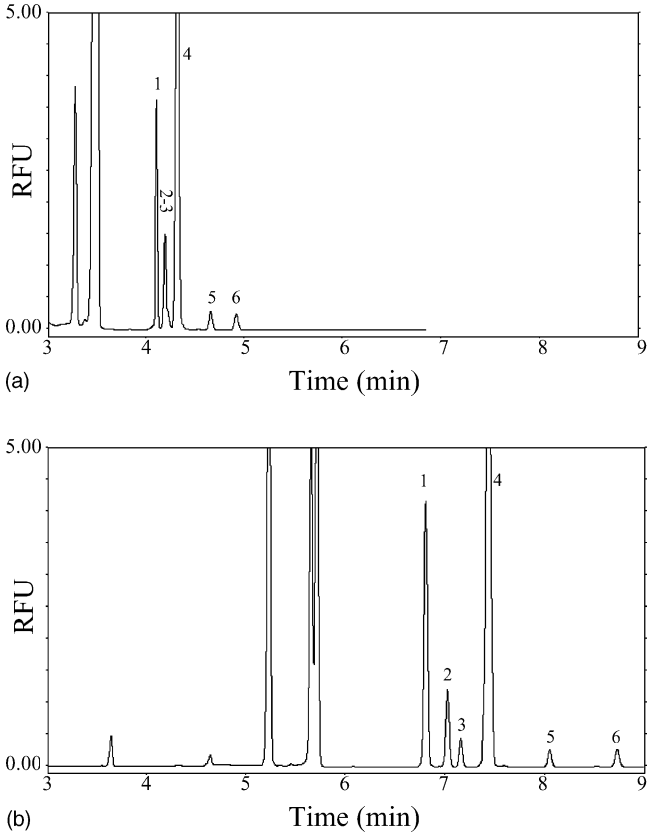

Fig. 2. Standard thiols analyzed in a 5 mmol/l sodium phosphate, 4 mmol/l boric acid, 75 mmol/l *N*-methyl-D-glucamine, at pH 11 run buffer (a) and in a 18 mmol/l sodium phosphate, 14.5 mmol/l boric acid with 75 mmol/l *N*-methyl-D-glucamine, at pH 11.4 (b). Electrophoretic conditions: 22 kV, 150 μA, 40 °C at normal polarity. Peak 1, cysteinylglycine (40 μmol/l); peak 2, D-Pen (10 μmol/l); peak 3, homocysteine (6 μmol/l); peak 4, cysteine (200 μmol/l); peak 5, glutathione (6 μmol/l); and peak 6, glutamylcysteine (6 μmol/l).

resolution. Therefore, we chose to extend the run time by incrementing the electrolyte run buffer concentration. As shown in Fig. 2b, a baseline resolution between all peaks was achieved when a 18 mmol/l sodium phosphate, 14.5 mmol/l boric acid with 75 mmol/l *N*-methyl-D-glucamine, at pH 11.4 run buffer were used, with a run time shorter than 9 min. *N*-Methyl-D-glucamine was nevertheless necessary, in fact by decreasing its concentration in the run buffer a loss of resolution between peaks was observed (data not shown).

3.2. Reproducibility, linearity, analytical recovery and sensitivity

Injection reproducibility was calculated by injecting the same sample 10 times consecutively. Within-run precision (intrassay) of the method was evaluated by injecting the same plasma sample 10 times consecutively, whereas between-run (interassay) precision was determined by injecting the same plasma sample for 10 consecutive days. Precision tests indicate a good repeatability of our method both for total D-Pen (migration times CV < 0.6%, peak areas CV < 2.8%, intrassay CV < 3.5%, interassay CV < 5.2%), for protein-bound D-Pen (migration times CV < 0.8%, peak areas CV < 3.9%, intrassay CV < 4.9%, interassay CV < 5.8%), for reduced D-Pen (migration times CV < 0.6%, peak areas CV < 3.7%, intrassay CV < 4.4%, interassay CV < 5.5%), for free D-Pen (migration times CV < 0.6%, peak areas CV < 3.2% intrassay CV < 4.0%, interassay CV < 5.4%). As seen in Table 1, calibration curves for different forms of penicillamine, by five replicate determinations, show good correlation coefficients, ensuring a linear response over the concentrations tested. D-Pen recovery was determined by adding authentic standards to plasma samples. In particular, we employed the plasma sample of a D-Pen administered Wilson's subject and analytical recovery of total and oxidized forms have been evaluated by adding oxidized thiols standards, while for the reduced form we supplemented the sample with reduced standard thiols. Sample was not incubated but immediately processed for thiols quantification. The analytical recoveries, evaluated at four different concentrations for each drug form, were 98.8–101.3 for total form, 98.6–100.8 for free form and 98.2–99.1 for reduced form (Table 2). The minimum detectable concentration, calculated by 14 nl injections of a known solution of standards (after 100-fold water dilution), was 200 pmol/l, corresponding to an injected quantity of 2.5 amol, for total, protein-bound and free penicillamine and 100 pmol/l, corresponding to an injected quantity of 1.5 amol, for reduced penicillamine, with a signal-to-noise ratio of 3. The limit of quantification (LOQ) measured in plasma was about 30 nmol/l for all thiols forms.

Table 1
Calibration curves of standard D-penicillamine obtained with the different procedures

Calibration range (μmol/l)	Procedure	Slope (CV%)	Intercept	r^2
2–48	1	1.763 (2.15)	0.5823	0.9994
2–24	2	1.448 (2.98)	0.3982	0.9991
0.4–8	3	0.583 (5.43)	0.0923	0.9981
0.1–2	4	0.969 (4.92)	0.0391	0.9959

Table 2
Recovery of the assay measured in a human plasma sample as mean of three replicate

D-Penicillamine form	Sample (μmol/l)	Added (μmol/l)	Measured (μmol/l)	Recovered (%)
Total	15.688	40	56.412	101.3
		20	35.402	99.2
		10	25.611	99.7
		5	20.440	98.8
Mean (CV%)				99.8 (1.09)
Free	0.660	1.6	2.244	99.3
		0.8	1.438	98.5
		0.4	1.068	100.8
		0.2	0.848	98.6
Mean (CV%)				99.3 (1.06)
Reduced	0.128	0.4	0.524	99.1
		0.2	0.323	98.9
		0.1	0.230	98.2
		0.05	0.176	99.0
Mean (CV%)				98.8 (0.41)

3.3. Plasma penicillamine concentrations

The new capillary electrophoresis method was applied to the determination of the redox status of plasma D-Pen from a Wilson's disease patient treated with an oral dose of 1.2 g/day of drug and the electropherograms obtained are shown in Fig. 3. Concentration of total D-Pen was 15.69 μmol/l and, as previously reported [34], the predominant form of D-Pen was bound to plasma protein (14.98 μmol/l) while concentrations of free oxidized and reduced forms were very low (0.532 and 0.128 μmol/l) probably because reduced penicillamine was extremely reactive and free disulphides are rapidly excreted in the urine, causing a rapid loss of this fractions [30]. Moreover, as reported by other authors [17,18,35], we observe that Wilson's disease patient treated with D-Pen has lower levels of total homocysteine and cysteine (3.8 and 145 μmol/l, respectively) when compared with normal subjects (10.6 and 247 μmol/l) and lower concentration of protein-bound form of homocysteine and cysteine (2.64 and 57 μmol/l) versus 8.4 and 142 μmol/l of healthy subjects (data not shown). These finding suggest that D-Pen treatment removes a considerable quantity of homocysteine and cysteine principally from the protein-bound fraction. After prolonged administration, D-Pen induces undesirable

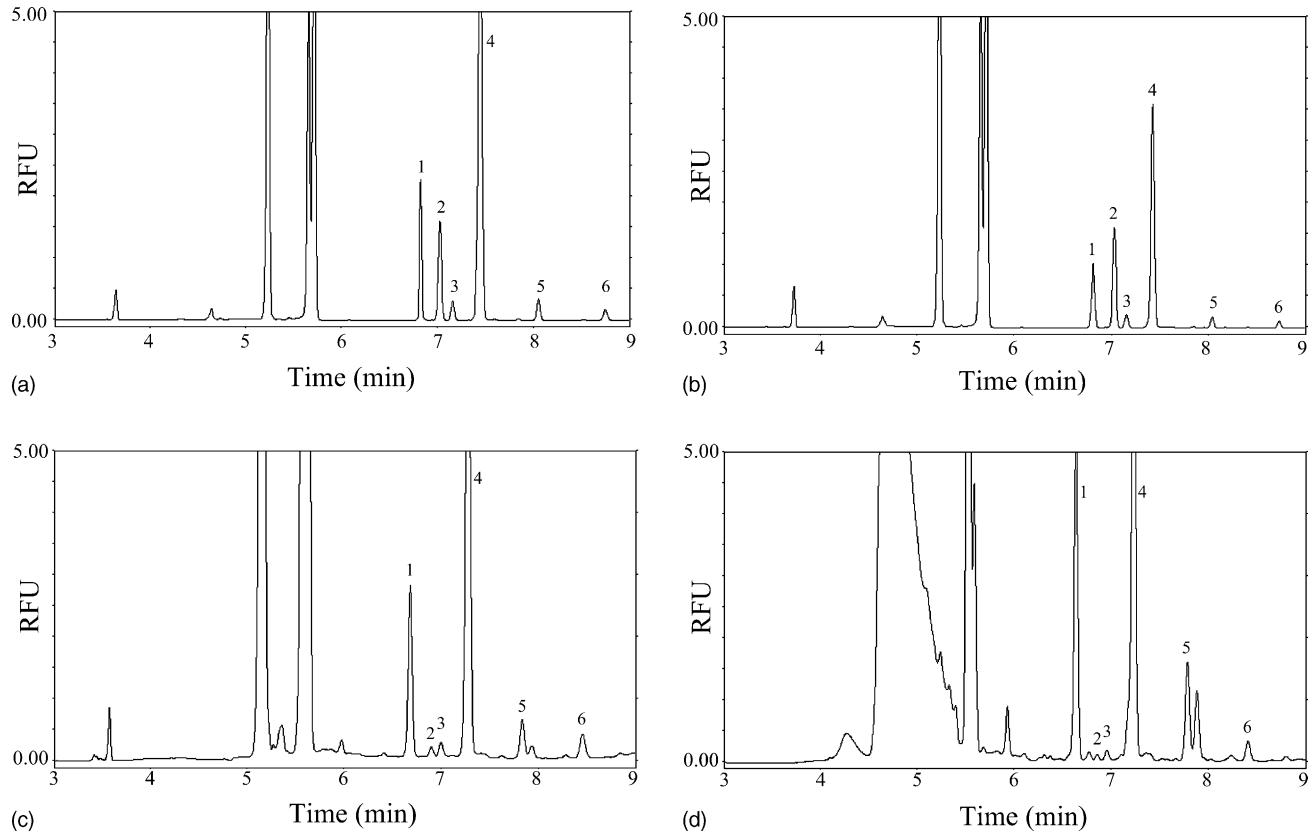

Fig. 3. Plasma D-Pen redox status in a Wilson's disease subject treated with an oral dose of 1.2 g/day of drug: procedure 1 (a): cysteinylglycine (20.9 μmol/l), D-Pen (15.69 μmol/l), homocysteine (3.84 μmol/l), cysteine (145.3 μmol/l), glutathione (7.27 μmol/l), glutamylcysteine (3.74 μmol/l); procedure 2 (b): cysteinylglycine (8.85 μmol/l), D-Pen (14.98 μmol/l), homocysteine (2.63 μmol/l), cysteine (57.1 μmol/l), glutathione (2.06 μmol/l), glutamylcysteine (1.44 μmol/l); procedure 3 (c): cysteinylglycine (12.13 μmol/l), D-Pen (0.66 μmol/l), homocysteine (1.20 μmol/l), cysteine (88.3 μmol/l), glutathione (5.20 μmol/l), glutamylcysteine (2.30 μmol/l); procedure 4 (d): cysteinylglycine (7.14 μmol/l), D-Pen (0.13 μmol/l), homocysteine (0.33 μmol/l), cysteine (17.5 μmol/l), glutathione (2.87 μmol/l), and glutamylcysteine (0.70 μmol/l).

side effects such as hypersensitivity, nephrotic syndrome, myasthenia gravis and increased elimination of essential elements although several case of prolonged administration without important toxic effects have been reported [1]. To evaluate the therapeutic effectiveness and the degree of possible toxicity research, calls for new methods able to measure the level of D-Pen in plasma and particularly to discriminate between oxidized (protein-bound and free oxidized) and reduced D-Pen that is the active form of drug. Many methods have been proposed in literature to measure total plasma D-Pen but none of these are able to measure different forms of D-Pen. By modifying our previous method, we developed the first capillary electrophoresis method to quantify reduced, free, protein-bound and total D-Pen in human plasma. Evidence for method applicability was obtained by assessing the different forms of the drug in a D-Pen administered Wilson's disease subject. Moreover, this method allows to quantify simultaneously the physiological thiols such as homocysteine, cysteine, glutathione, glutamylcysteine and cysteinylglycine so that relationships between drug administration and plasma thiols concentration could be also evaluated.

Acknowledgements

This study was supported by the "Assessorato dell'Igiene e Sanità Regione Autonoma della Sardegna", by the "Ministero dell'Istruzione, dell'Università e della Ricerca" and by the "Ministero della Sanità (Attività di Ricerca Finalizzata—2002)" (Italy). The manuscript language revision by the Ms. Maria Antonietta Meloni is greatly appreciated.

References

[1] J.M. Walshe, Mov. Disord. 18 (2003) 853.
[2] D.A. Joyce, Baillieres Clin. Rheumatol. 4 (1990) 553.
[3] C.R. Chong, D.S. Auld, Biochemistry 39 (2000) 7580.
[4] J.M. Walshe, Am. J. Med. 21 (1956) 487.
[5] B. Sarkar, Chem. Rev. 99 (1999) 2535.
[6] S.R. Gooneratne, D.A. Christensen, Vet. J. 153 (1997) 171.
[7] E.C. LeRoy, M. Trojanowska, E.A. Smith, Clin. Exp. Rheumatol. 9 (1991) 173.
[8] I.A. Jaffe, Am. J. Med. 75 (1983) 63.
[9] A.D. Stephens, J. Inherit. Metab. Dis. 12 (1989) 197.
[10] J. Peisach, W.E. Blumberg, Mol. Pharmacol. 5 (1969) 200.
[11] C.M. Herbert, K.A. Lindberg, M.I. Jayson, A.J. Bailey, Lancet 303 (1974) 187.

[12] W.H. Deutsch, J.F. Morton, Science 125 (1957) 600.
[13] R.R. Singh, A.N. Malaviya, R. Malaviya, M. Sharma, R. Shandilya, V. Taneja, S.D. Khare, D. Beri, A. Kumar, N.K. Mehra, J. Assoc. Phys. India 42 (1994) 201.
[14] R. Munro, H.A. Capell, Br. J. Rheumatol. 36 (1997) 104.
[15] D.J. Perrett, Rheumatol. Suppl. 7 (1981) 41.
[16] W.F. Kean, N. Bellamy, C.J. Lock, J. Adachi, Clin. Invest. Med. 7 (1984) 77.
[17] S.S. Kang, P.W. Wong, K. Curley, Pediatr. Res. 16 (1982) 370.
[18] S.S. Kang, P.W. Wong, P.B. Glickman, C.M. MacLeod, I.A. Jaffe, J. Clin. Pharmacol. 26 (1986) 712.
[19] P.R. Pal, J. Biol. Chem. 234 (1959) 618.
[20] P. Vermeit, Pharm. Weekbl. 112 (1977) 130.
[21] J. Mann, P.D. Mitchell, Am. J. Pharm. Pharmacol. 31 (1979) 420.
[22] E.S.K. Assem, Curr. Med. Res. Opin. 2 (1974) 568.
[23] E.S.K. Assem, M.R. Vickers, Postgrad. Med. J. 50 (1974) 65.
[24] A.O. Muijsers, R.J. Van de Stadt, A.M. Henrichs, J.K. Van der Korst, Clin. Chim. Acta 94 (1979) 173.
[25] J. Russell, J.A. McKeown, C. Hensman, W.E. Smith, J. Reglinski, J. Pharm. Biomed. Anal. 15 (1997) 1757.
[26] P.M. Brooks, M.W. Whitehouse, J. Chromatogr. 275 (1983) 89.
[27] M. Yusof, R. Neal, N. Aykin, N. Ercal, Biomed. Chromatogr. 14 (2000) 535.
[28] F. Freuzig, J. Frank, J. Chromatogr. 218 (1981) 615.
[29] D.A. Joyce, D.N. Wade, J. Chromatogr. 430 (1988) 319.
[30] G.T. Yamashita, D.L. Rabenstein, J. Chromatogr. 491 (1989) 341.
[31] N. Kucharczyk, S. Shahinian, J. Rheumatol. 7 (1981) 28.
[32] J. Russell, D.L. Rabenstein, Anal. Biochem. 242 (1996) 136.
[33] A. Zinellu, C. Carru, F. Galistu, M.F. Usai, G.M. Pes, G. Baggio, G. Federici, L. Deiana, Electrophoresis 24 (2003) 2796.
[34] P. Netter, B. Bannwarth, P. Pere, A. Nicolas, Clin. Pharmacokinet. 13 (1987) 317.
[35] P.M. Ueland, H. Refsum, Nord. Med. 104 (1989) 293.