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A novel HPLC-electrochemical detection approach for the determination of D-penicillamine in skin specimens

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ABSTRACT

D-penicillamine is a thiol drug mainly used for Wilson's disease, rheumatoid arthritis and cystinuria. Adverse effects during normal use of the drug are frequent and may include skin lesions. To evaluate its toxic effects in clinical cases an original method based on high performance liquid chromatography coupled to amperometric detection in a specific biological matrix such as skin has been developed.

The chromatographic analysis of D-penicillamine was carried out on a C18 column using a mixture of acid phosphate buffer and methanol as the mobile phase. Satisfactory sensitivity was obtained by oxidizing the molecule at +0.95 V with respect to an Ag/AgCl reference electrode. A chemical reduction of D-penicillamine-protein disulphide bonds using dithioerythritol combined with microwaves was necessary for the determination of the total amount of D-penicillamine in skin specimens. A further solid-phase extraction procedure on C18 cartridges was implemented for the sample clean-up. The whole analytical procedure was validated: high extraction yield (> 91.0%) and satisfactory precision (RSD < 6.8%) values were obtained. It was successfully applied to skin samples from a patient who was previously under a long-term, high-dose treatment with the drug and presented serious D-penicillamine-related dermatoses. Thus, the method seems to be suitable for the analysis of D-penicillamine in skin tissues.

1. Introduction

Penicillamine (2-amino-3-methyl-3-sulfanyl-butanoic acid) is a sulphur-containing amino acid that belongs to the aminothiol family. It can exist in D and L enantiomeric forms (Fig. 1); however, because of the toxicity of the levorotatory isomer, the racemic mixture has been replaced for clinical purposes by purified D-penicillamine (D-Pen, Fig. 1a) [1,2]. D-Pen is used as a chelating agent in the treatment of Wilson's disease, a rare autosomal recessive genetic disorder of copper transport; as an antifibrotic agent to treat scleroderma; and as immunosuppressant drug to treat patients with active rheumatoid arthritis [3,4]. Moreover, it is an efficient antidote to heavy metal poisoning and has been frequently used for the treatment of cystinuria, an inherited disorder of renal excretion of cystine and other dibasic amino acids [4]. After oral administration, D-Pen is rapidly but variably (50–70%) absorbed from the gastrointestinal tract and it is reported to be more than 80% bound to plasma proteins; then, it is oxidized to various disulphide forms. Elimination is biphasic with an initial phase of 1–3 h followed by a second slower phase due to a gradual release from tissues [5]. About 50% of patients experienced one or more adverse effects of D-Pen such as anorexia, loss of taste, oral ulceration, skin rashes, haematological effects and glomerulonephritis [4]. Skin adverse events related to D-Pen are common, occurring in 25–50% of patients [6,7]. In fact, it can cause cutaneous elastin and collagen abnormalities, such as pseudo-pseudoxanthoma elasticum (p-PXE), elastosis perforans serpiginosa (EPS), acquired cutis laxa and anethoderma. The spectrum of elastic and collagen tissue disorders ascribed to D-Pen treatment is clinically indistinguishable from the idiopathic forms; on the contrary histological D-Pen-induced changes create a specific pathological pattern [7]. However, the pathogenesis of the D-Pen-induced degenerative dermatoses is still not well defined, even if some theories (such as the indirect inhibition of copper-dependent enzyme required for the production of the elastin crosslinks or the blockage of the aldehyde crosslink precursors), based on specific histopathologic and electron

Abbreviations: D-Pen, D-penicillamine; IS, internal standard; DTE, dithioerythritol; SPE, solid phase extraction; EPS, elastosis perforans serpiginosa; p-PXE, pseudo-pseudoxantoma elasticum; RSD, relative standard deviation; LOQ, limit of quantitation; LOD, limit of detection; SD, standard deviation

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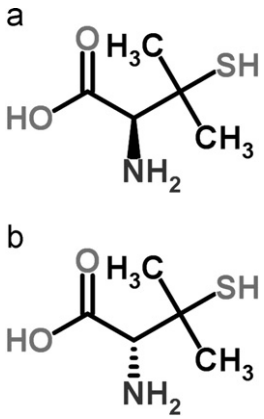


Fig. 1. Chemical structures of (a) D-Pen; and (b) L-Pen.

microscopic findings, have been hypothesised [8]. To our knowledge the actual presence of the drug in skin lesions has not been yet shown. Thus, our aim is the development of a reliable analytical method to identify and quantify D-Pen in skin samples from patients who had received this drug in a long term treatment and showed typical features of D-Pen-induced elastosis.

Most of the analytical techniques reported in the literature for the measurements of D-Pen in biological matrices are dating back to the first years after the discovery and therapeutic applications of the drug. Some methods include liquid chromatography (HPLC) with diode array [9] or spectrofluorimetric [10,11], or electrochemical [12–14] or chemiluminescence [15] detector; a few papers are related to capillary electrophoresis [16–18]. The previous papers usually concerned with human plasma [12,13,17,18], urine [9,10,12,15] and synovial [13] fluids as well as mouse tissues [11,14]. Herein, an original HPLC method based on the use of an amperometric detector for the determination of D-Pen in skin is presented. In comparison with our method, the chromatographic methods which used electrochemical detection [12–14] were less sensitive or partial validated [13] or employed ion-exchange resin as stationary phase [12] less reliable than reversed-phase ones. The proposed method has been successfully applied to skin samples from a patient who was previously under a long-term, high-dose treatment with D-Pen (used as a copper chelator of Wilson's disease) and presented both p-PXE and EPS dermatoses 25 years after the drug discontinuation.

2. Material and methods

2.1. Chemicals

D-Pen, 2,3-dihydroxybenzoic acid (used as the internal standard, IS), 85.0% (w/w) phosphoric acid, potassium dihydrogen phosphate, 0.6 N trichloroacetic acid (TCA), 2 N sodium hydroxide, methanol for HPLC, potassium chloride (KCl), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), 1-octanesulfonic acid (OSA) and dithioerythritol (DTE) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm), obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA), was used for the preparation of all the solutions.

2.2. Preparation of solutions

Primary stock solutions of D-Pen (1.0 mg mL^{-1}) were prepared by dissolving 20.0 mg of pure substance in 20.0 mL of 0.1% EDTA solution (see below); the IS stock solutions (1.0 mg mL^{-1}) were prepared by dissolving 20.0 mg of pure substance in 20.0 mL of methanol. Working standard solutions at different concentrations were obtained freshly every day by diluting primary stock solutions with ultrapure water. Stock solutions were stable for at least three weeks when stored at −20 °C.

The saline (0.9% NaCl) and the 0.1% EDTA solutions were prepared by dissolving 450 mg of NaCl and 50 mg of EDTA in 50 mL of ultrapure water, respectively; the 50 mM DTE solution was obtained by dissolving 7.71 mg of DTE in 1 mL of ultrapure water.

2.3. Apparatus and chromatographic conditions

The chromatographic apparatus consisted of a Varian (Harbor City, CA, USA) 9002 chromatographic pump and an Antec (Leiden, The Netherlands) Decade II amperometric detector, equipped with a cell with a glassy carbon working electrode and an Ag/AgCl reference electrode. The analytical cell was set at a potential of +0.95 V. Data processing was handled by means of a Varian Star Chromatography software. The chromatographic separation was achieved by isocratic elution on a Thermo Scientific (Waltham, MA, USA) Hypersil Gold reversed-phase column (C18, 150 × 4.6 mm i.d., 5 μm), based on highly pure silica with specific modification. The mobile phase was a mixture (11.5:88.5, v/v) of methanol and an aqueous solution of phosphate buffer (pH 2.5; 16.4 mM), 254.2 mg L^{-1} OSA and 33.9 mg L^{-1} EDTA. The mobile phase was filtered through a Phenomenex (Torrance, CA, USA) membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic apparatus. The flow rate was 1.0 mL min^{-1}. The samples were injected into the HPLC system by means of a 50 μL loop. A Crison (Barcelona, Spain) MicropH 2000 pHmeter, an Universal 32 R centrifuge from Hettich (Tuttlingen, Germany), an IKA (Milan, Italy) HB 10 Basic rotary evaporator, a household microwave oven (Bologna, Italy) and a vortex agitator were also used.

2.4. Skin tissue collection and processing

Skin tissues were obtained from a fasting 57-year-old white woman, who in the past had been treated with D-Pen in large (average daily dosage of 2 g) as therapy for Wilson's disease for 17 years. Then, the drug was discontinued and after 25 years the patient had received a histological diagnosis of two D-Pen-associated dermatoses, namely elastosis perforans serpiginosa (EPS) and pseudo-pseudoxanthoma elasticum (p-PXE). Two 4 mm punch biopsies were taken from skin areas affected by p-PXE (neck) and EPS (groin), respectively. Skin samples were stored in suitable tubes at −80 °C until the analysis, when tissues (0.03 g of wet weight) were washed with saline solution to remove blood, minced, and, then homogenized in 100 μL of 0.1% EDTA (2 min). After adding 80 μL of phosphate buffer (0.05 mM; pH = 8.5), 50 μL of DTE and 20 μL of IS solutions, a microwave treatment (350 W, 6 min) was performed to allow the reduction of –SS-bounds; then the mixture was treated with 100 μL of 0.6 N TCA solution and centrifuged (4000 rpm or 1780 × *g*, 5 °C, 2 min). The supernatant was subjected to further extraction and clean-up steps (see below).

Skin samples from seven fasting healthy volunteers (used as blank samples), who were not subjected to the treatment with D-Pen, were processed in the same way.

2.5. Solid phase extraction procedure

Solid-phase extraction (SPE) for the sample pre-treatment was carried out using IST (Hengoed, UK) Isolute C18 cartridges (100 mg, 1 mL) by means of a Varian VacElut apparatus. The cartridges were activated with 5 × 1 mL of methanol and conditioned with 5 × 1 mL of phosphate buffer (0.01 M; pH = 3.0).

The supernatant obtained after the process previously described (see Section 2.4) was loaded onto a previously conditioned C18 cartridge. After loading, the cartridges were washed with 0.5 mL of phosphate buffer (0.01 M; pH=3.0). Elution was carried out with 1 mL of methanol. The eluate was dried under a vacuum (rotary evaporator), re-dissolved in 200 μL of ultrapure water, and injected into the HPLC system.

2.6. Method validation

Method validation procedures were carried out according to official guidelines [19,20].

2.6.1. Extraction yield (absolute recovery) and precision

Aliquots of 20 μL of D-Pen standard solutions at three different concentrations (in order to obtain on-column concentrations of 5, 250 and 500 ng mL^{-1}, respectively), containing the IS at a constant concentration (in order to obtain on-column concentration of 25 ng mL^{-1}), were added to blank skin samples (0.03 g) after homogenizing with EDTA solution. The homogenized samples were subjected to the procedures described in Sections 2.4. and 2.5, then injected into the HPLC. The analyte peak area was compared to those obtained injecting standard solutions at the same theoretical concentrations and the absolute recovery was calculated.

The assays described above were repeated six times within the same day to obtain repeatability (*intraday precision*) and six times over six different days to obtain intermediate precision (*interday precision*), both expressed as percentage relative standard deviation values (RSD%).

2.6.2. Calibration curves, limit of quantitation and limit of detection

Aliquots of 20 μL of D-Pen standard solutions at seven different concentrations (in order to obtain on-column concentrations of 5, 100, 125, 250, 300, 400 and 500 ng mL^{-1}, respectively), containing the IS at a constant concentration, were added to blank skin samples (0.03 g) after homogenizing with EDTA solution. The homogenized samples were subjected to the procedures described above and, then injected into the HPLC. The analyte/IS peak area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}). The calibration curves were constructed by means of the least-square method. The values of limit of quantitation (LOQ) and limit of detection (LOD) were calculated as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.6.3. Selectivity

The method selectivity was evaluated by injecting into HPLC system standard solutions of several endogen compounds, such as thiols and disulphides (concentration level=100 ng mL^{-1}).

Blank skin samples derived from seven healthy volunteers not receiving any pharmacological therapy were subjected to the sample pre-treatment procedure and injected into the HPLC system. The resulting chromatograms were checked for possible interference from endogenous compounds. The acceptance criterion was no interfering peak higher than an analyte peak corresponding to its LOD.

2.6.4. Stability

Stability was tested in stock solutions of D-Pen by comparing the chromatographic peak area ratios (analyte to internal standard) of a standard solution of 250 ng mL^{-1} from stock solution stored for three weeks at −20 °C with those obtained from the fresh stock sample (n=3).

Stability assays were also carried out in skin samples spiked with a known amount of D-Pen (i.e. on column concentration of 250 ng mL^{-1}) and stored for 24 h at room temperature as well as for up to three freeze-thaw cycles. The peak area ratios (analyte to internal standard) of extracted volunteer skin samples were compared with those of aliquots of the same samples when fresh, after SPE procedure (n=3).

2.6.5. Accuracy

The accuracy of the method was evaluated by means of recovery studies. Appropriate amounts of the analyte (corresponding to on-column concentration of 50 and 100 ng mL^{-1}) were added to skin samples containing known amounts of D-Pen (i.e. previously analysed samples). The spiked samples (n=2) were submitted to the SPE procedure and analysed. Recovery values were calculated according to the following formula: 100 × ([after spiking] − [before spiking])/[added].

3. Results and discussion

3.1. Choice of the chromatographic conditions

To achieve the optimum chromatographic performance, preliminary assays were carried out by testing some kinds of stationary phases (C8, C18 and pentafluorophenyl) coupled with a mobile phase composed of acid phosphate buffer and methanol. Considerable differences among columns were found and Hypersil Gold C18 column provided the best results in terms of the peak shape and sensitivity. On the basis of the chromatographic behaviour of the eluted compounds (i.e. D-Pen, IS and the reduced and oxidized forms of DTE, used as the reducing agent), methanol percentage, concentrations of OSA (as an ion-pairing agent in the mobile phase) and the pH values of buffer were examined for optimizing the elution conditions. Using 11.5% methanol coupled with the phosphate buffer at pH value of 2.5 and OSA in the concentration of 254.2 mg L^{-1}, D-Pen and IS (2,3-dihydroxybenzoic acid) were successfully resolved without any interference. The next step was to find the best electrochemical conditions for improving the sensitivity of the amperometric detector. A range of working potentials from 0.6 V to 1.2 V were investigated; good results in terms of signal to noise ratio were obtained by applying a working potential of +0.95 V at glassy carbon electrode vs the Ag/AgCl reference one.

Fig. 2a shows a typical chromatogram of a standard solution containing D-Pen and IS obtained under the optimized conditions. As can be seen, the peaks are neat and well-resolved and the chromatographic separation is within 16.0 min.

3.2. Reduction of D-Pen-protein disulphide bonds

Because it is hypothesised that D-Pen is concentrated in the skin as D-Pen-protein disulphides, measurements of total analyte involves the reduction of these disulphide bonds and the detection of the generated D-Pen. Dithioerythritol ($HS–CH_2(CHOH)_2CH_2–SH$) is capable of maintaining monothiols completely in the reduced state and of reducing disulphides quantitatively because of its low redox potential (−0.33 V) [21]. The reduction of a typical disulphide bond ($R_1–SS–R_2$) proceeds by two sequential thiol-disulphide exchange reactions:

$$R_1–SS–R_2 + HS–CH_2(CHOH)_2CH_2–SH \rightleftarrows R_1–SH + R_2–SS–CH_2(CHOH)_2–SH \quad (1)$$

The reduction usually does not stop at the mixed-disulphide species because the second thiol of DTE ($R_2–SS–CH_2(CHOH)_2–SH$) has a high propensity to close the ring, forming oxidized DTE and

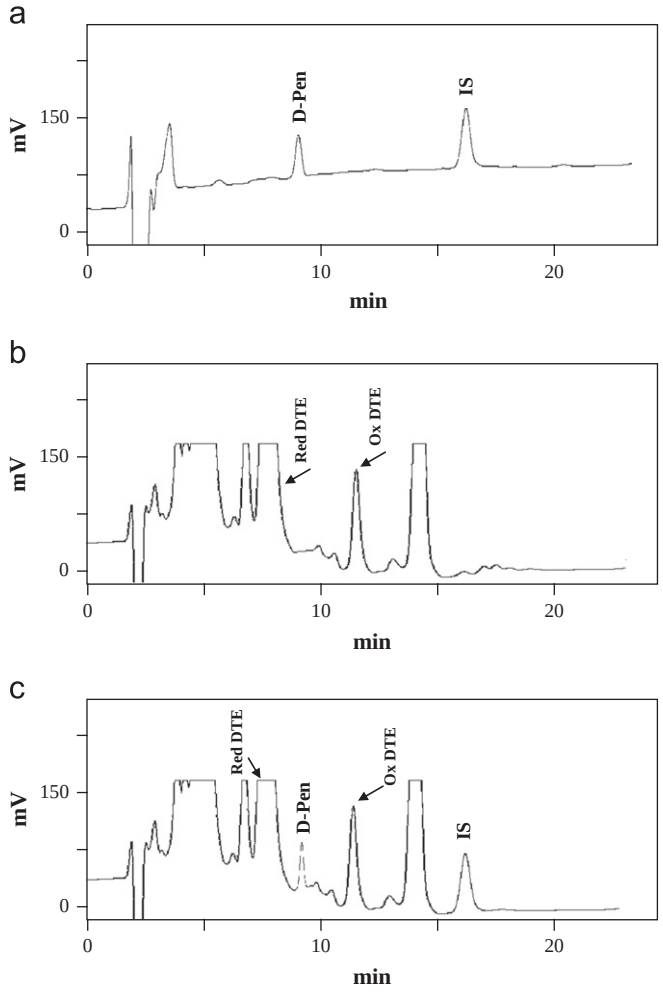
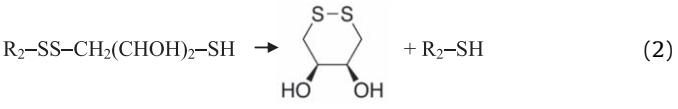


Fig. 2. Chromatograms of (a) a standard solution containing 250 ng mL^{-1} of D-Pen and 25 ng mL^{-1} of IS; (b) a blank skin sample; and (c) the same skin sample spiked with 250 ng mL^{-1} of D-Pen and 25 ng mL^{-1} of IS (on-column concentrations). Experimental conditions are described in Section 2. Red DTE, reduced DTE; Ox DTE, oxidized DTE.

leaving behind a reduced disulphide bond (R_2–SH):



(2)

The reducing power of DTE is limited to pH values above 7, since only the negatively charged thiolate form $–S^-$ is reactive. However, even DTE cannot reduce buried disulphide bonds, so the reduction should be carried out under strong conditions (e.g., at high temperatures or in several hours at pH > 7). The severe drawback is represented by very long reaction times (usually 10–12 h), which cannot be shortened by excessive heating, otherwise degradation would occur. We tried a microwave treatment instead of the usual oven heating in order to speed up the whole process with DTE. For this purpose, different reaction times in the 1–8 min range were tested, at full instrumental power (350 W). The employment of a microwave oven in the presence of a phosphate buffer at pH 8.5 allowed us to obtain the best results in terms of reduced disulphide bonds within 6 min. Degradation of D-Pen in standard solution, containing the analyte (at a concentration of 250 ng mL^{-1}), was investigated over the treatment time with microwaves: the chromatographic peak area and shape of D-Pen before and after the treatment were compared. No signals of degradation were evident.

3.3. Development of the skin sample clean-up procedure

The determination of drugs in biological matrices involves a preliminary treatment, since proteins and other biological compounds may result in precocious deterioration of the performance of chromatographic columns and in increased column backpressure. The analysis of a drug in skin samples is particularly complex, as it is poorly described into literature and shows several critical points which should be accurately evaluated to obtain reliable results. Initially, it was decided to carry out the precipitation of proteins by means of TCA which, by itself, however, did not provide an adequate clean-up of the homogenized skin samples. Therefore, an SPE procedure was added. Different SPE sorbents were tested: C8, C18, diol, cyano, Oasis HLB (hydrophilic/lipophilic balance), Oasis MAX (mixed-mode: HLB and anion exchange) or SAX (strong anion exchange). The C18 sorbent allowed higher extraction yields (> 90%) as well as a good matrix clean-up than other tested sorbents (Fig. 3a).

Loading, washing and elution steps were then examined. In particular, an acid phosphate buffer was used to allow the complete retention of the analyte onto the cartridge. Moreover, different solutions were considered in the washing step: ultrapure water, acid phosphate buffer and mixtures of ultrapure water and methanol (from 5% to 15%) as well as phosphate buffer and methanol (from 5% to 10%). The last ones guaranteed better matrix clean-up, but worsened the extraction yields, resulting always less than 50% (Fig. 3b). So, it was decided to use 100% acid

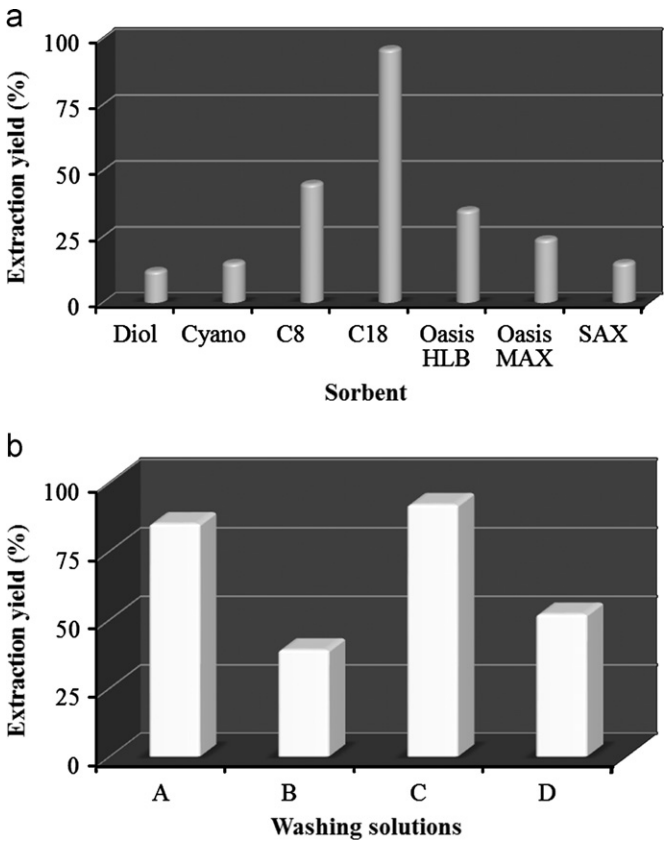


Fig. 3. Mean extraction yields (%) of D-Pen as a function of (a) kind of sorbent and (b) washing solution. Experimental conditions are described in Section 2. *A*,ultrapure water; *B*, ultrapure water/methanol (85:15, v/v); *C*, acid phosphate buffer; *D*, acid phosphate buffer/ methanol (90:10, v/v).

phosphate buffer for the washing step. Pure methanol (1 mL) was selected as the elution solvent which allowed obtaining extraction yields always greater than 90%.

The chromatograms of a blank skin sample from a healthy volunteer and of the same sample spiked with D-Pen and the IS show no interference from the matrix (Fig. 2b and c).

3.4. Method validation

The calibration curve exhibited a good linear behaviour over the 5–500 ng mL^{-1} concentration range. Typical calibration line was expressed as $y=0.0035x-0.0012$ ($r_c=0.9992$), where y is the peak area ratio and x is the concentration in ng mL^{-1}. The LOQ and LOD values were 5 ng mL^{-1} and 1.5 ng mL^{-1} (on-column concentrations), respectively.

Extraction yield and precision assays were carried out at three different concentration levels of D-Pen, corresponding to the lowest level, highest level and middle point of calibration curve. The results in terms of extraction yield were satisfactory being always higher than 91.0%. The mean extraction yield of the IS was 93.0% (standard deviation or SD= ± 2.5). The precision was also satisfactory with RSD value always lower than 6.8% (Table 1).

3.5. Selectivity

The selectivity of the method was evaluated by injecting standard solutions of some endogenous compounds, such as thiols (e.g., glutathione, cysteine, homocysteine) and disulphides (glutathione disulphide, cystine). None of these compounds interfered with the determination of D-Pen. The analysis of blank skin samples from seven subjects, who did not receive any pharmacological treatment, was carried out; no interference at the retention time of analyte and IS was observed.

3.6. Stability

The mean difference in peak area ratios (analyte to internal standard) between a standard solution of D-Pen (250 ng mL^{-1}) from a stock solution after storage and the standard solution from a freshly prepared stock solution was −1.2% (SD= ± 0.3), indicating that D-Pen was stable in the EDTA solution when stored at −20 °C for at least 3 weeks.

As regard skin samples the mean difference in peak area ratios (analyte to internal standard) was −0.5% (SD= ± 0.1) and – 0.7% (SD= ± 0.2) when the biological samples were stored at room temperature for 24 h and for up to three freeze-thaw cycles, respectively.

3.7. Application to patient skin tissues

The validated analytical method was applied to the analysis of D-Pen in skin samples from a patient presenting both PXE and EPS dermatoses, probably attributable to the previous D-Pen long-term treatment. The particularity of the clinic case was that the skin D-Pen-related dermatoses appeared 25 years after the drug discontinuation and were mainly localized on the neck and groin. At present the patient is under zinc acetate treatment for Wilson's disease. The chromatogram of a skin sample (processed as previously described) is reported in Fig. 4. As one can see, a chromatographic peak corresponding to D-Pen is evident at 8.9 min, the retention time of the analyte. The concentration of D-Pen was found to be 191 ± 7 ng mL^{-1} (corresponding to a skin concentration of 638 ± 5 ng g^{-1}).

Table 1
Validation parameters.

Analyte	Amount added (ng mL^{-1})[a]	Extraction yield (%)[b]	Repeatability (RSD%)[b]	Interday precision (RSD%)[b]
D-Pen	5.0	91.1	6.56	6.77
	250.0	93.8	6.15	6.25
	500.0	95.4	6.12	6.24

[a] On column concentration.
[b] $n=6$.

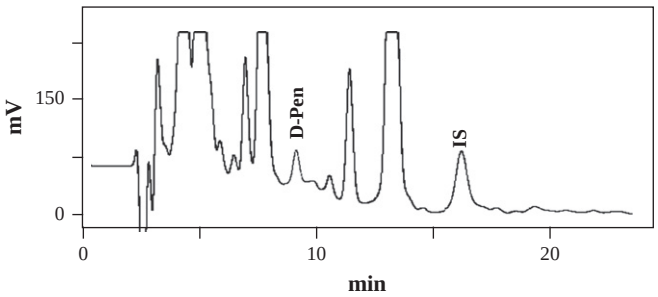


Fig. 4. Chromatogram of a skin sample from a female patient who showed serious dermatological diseases 25 years after D-Pen discontinuation.

3.7.1. Accuracy

Accuracy was evaluated by means of recovery assays. Patient skin samples were analysed, then known amounts of D-Pen were added to the same skin samples, which were analysed again. The recovery of the added D-Pen was then calculated. The mean recovery value was 88% (SD= ± 3), thus accuracy was satisfactory.

4. Conclusion

A reliable method based on the use of an HPLC system with amperometric detection combined with an SPE procedure for the analysis of D-Pen in skin samples has been developed. Good results in terms of extraction yield (> 91%), precision (RSD < 6.8%), sensitivity (LOQ=5.0 ng/mL), accuracy (mean value of 88%) and selectivity were obtained. To our knowledge, no paper dealing with the analysis of D-Pen in skin has been found in the literature. The figures of merit are better than those of previous methods [9–18] concerning the analysis of D-Pen in other biological matrices. Moreover, the present method is advantageous, since the quantitative reduction of D-Pen-protein-disulphides with DTE is carried out by means of microwaves so being very fast. It has been successfully applied to the analysis of some skin samples from a patient who showed both EPS and p-PXE diseases 25 years after D-Pen discontinuation and interesting results were obtained. The main novelty of the proposed method is the determination of D-Pen in skin tissues, which can be used to detect the presence of the drug in D-Pen-related dermatoses. In fact, so far, the onset of skin diseases such as EPS and p-PXE has always been based on histopathological and epidemiological data [8], but the actual presence of D-Pen in skin tissues has not ever shown.

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