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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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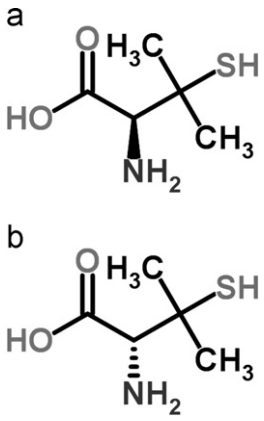

Fig. 1. Chemical structures of (a) D-Pen; and (b) L-Pen.

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).