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A direct LC/MS/MS method for the determination of ciclopirox penetration across human nail plate in *in vitro* penetration studies

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ABSTRACT

Due to severe chelating effect caused by N-hydroxylpyridone group of ciclopirox, there is no published direct HPLC or LC/MS/MS method for the determination of ciclopirox in any *in vitro* or *in vivo* matrix. Instead, the time-consuming pre-column derivatization methods have been adapted for indirect analysis of ciclopirox. After overcoming the chelating problem by using K_2EDTA coated tubes, a direct, sensitive and high-throughput LC/MS/MS method was successfully developed and validated to determine the amount of ciclopirox that penetrated across the nail plate during *in vitro* nail penetration studies. The method involved adding a chemical analog, chloridazon as internal standard (IS) in K_2EDTA coated tubes, mixing IS with ciclopirox in a 96-well plate and then proceeding to LC/MS/MS analysis. The MS/MS was selected to monitor m/z 208.0 → 135.8 and 221.8 → 77.0 for ciclopirox and IS, respectively, using positive electrospray ionization. The method was validated over a concentration range of 8–256 ng/mL, yielding calibration curves with correlation coefficients greater than 0.9991 with a lower limit of quantitation (LLOQ) of 8 ng/mL. The assay precision and accuracy were evaluated using quality control (QC) samples at three concentration levels. Analyzed concentrations ranged from 101% to 113% of their respective nominal concentration levels with coefficients of variation (CV) below 10.6%. The average recovery of ciclopirox from nail matrix was 101%.

The validated method was successfully used to analyze the ciclopirox formulation and *in vitro* nail penetration samples.

1. Introduction

Ciclopirox (6-cyclohexyl-1-hydroxyl-4-methyl-2(1*H*)-pyridone) (Fig. 1) is a synthetic broad-spectrum antifungal agent that inhibits the growth of dermatophytes, a type of fungus that grows on the skin, hair and nail [1,2]. The mechanism of action probably involves its chelating with polyvalent metal ions such as Fe^{3+} and Al^{3+} and thus inhibiting the metal-dependent enzymes within fungal cells [2]. Several ciclopirox formulations including cream, lotion, lacquer and gel have been developed to treat skin and nail infections, such as tinea pedis and onychomycosis [2,3]. Despite its good *in vitro* antifungal activity, the lack of robust clinical efficacy has focused attention on whether ciclopirox has adequate skin and nail penetration. With several *in vitro* models [4–6] developed for evaluations of the new chemical entity (NCE) and comparison with that of ciclopirox, there has been increased need for rapid and accurate determination of ciclopirox in *in vitro* study matrices, and it was the goal for us to develop a high-throughput LC/MS/MS method for direct analysis of ciclopirox in *in vitro* nail penetration matrix.

The N-hydroxylpyridone group in ciclopirox interacts strongly with trace metal ions in solvent and assay systems, and with silica gel based HPLC adsorbents through a chelating effect resulting severe chromatographic peak tailing and non-linear responses of peak area vs. ciclopirox concentration, which makes direct determination of ciclopirox challenge. Because of this strong complexation of ciclopirox with metal cations on the stationary phases of HPLC columns resulting irreversible retain of ciclopirox when small quantities presented and severe tailing when large amounts injected [9], ciclopirox cannot be directly quantified by either normal phase chromatography or reverse phase chromatography [9,14]. A modification of the stationary phase for reversed phase HPLC was reported for the determination of ciclopirox in antidandruff preparations [15]. In this method, the novel column after multi-step chemical modification and deactivation of the surface minimized silanol groups and heavy metal cation contents and therefore, reduced the chelating complexation [15]. A USP method [7] applied extended time washing (15 h) and equilibrating (5 h) before a new column could be used in the experiment to ensure the desorption of disruptive metal ions. However, the badly tailed

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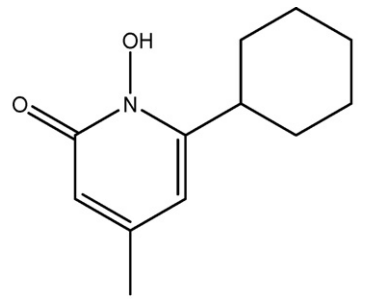
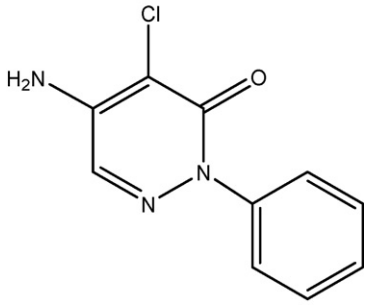


(I)



(II)

Fig. 1. Chemical structures of ciclopirox (I) and chloridazon (II).

peak and poor quantitation were still experienced [8]. Although the pre-column derivatization methods [5,9,13] eliminated the chelating effect by methylation of the N-hydroxyl group enabling the ciclopirox to be quantified, it involved a complicated and time-consuming procedure which created a bottleneck for the assays especially when large sample size was involved. A recently published micellar electrokinetic capillary chromatography (MECK) method demonstrated significant improvement of ciclopirox peak by utilizing EDTA as a chelating agent in the mobile phase and optimizing other key factors including buffer additive concentrations, pH, and applied voltage [8]. However, due to its low sensitivity with LOQ of 31.3 μg/mL this MECK method is unsuitable for trace level ciclopirox determination from *in vitro* nail penetration studies and other pharmacokinetic studies. The sensitivity (LOQ of 2 μg/mL) of the HPLC method [15] with modified column using EDTA in the mobile phases was also beyond the satisfaction of trace level determination of ciclopirox.

After overcoming the chelating effect by utilizing K_2EDTA tubes in the sample preparation, a simplistic, novel, direct, sensitive and high-throughput LC/MS/MS method for the determination of ciclopirox in *in vitro* nail penetration samples was developed. The method was validated and the *in vitro* nail penetration experimental samples and formulation stability samples were analyzed.

2. Experimental

2.1. Materials

Ciclopirox (I, Fig. 1) was a product of A.K. Scientific, Inc. (Mountain View, CA, USA). Penlac® nail lacquer (ciclopirox) topical solution, 8% was a prescription product of Dermik Laboratories (Berwyn, PA, USA), a division of Aventis. The BD Vacutainer* Venous Blood Collection Tubes 4-mL, 7.2 mg K_2EDTA was purchased from VWR (West Chester, PA, USA). Chloridazon (II, Fig. 1), ammonium hydroxide solution and formic acid (ACS reagent) were from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade water, methanol, acetonitrile and ammonium acetate were the products of Mallinckrodt Baker (Phillipsburg, NJ, USA). Dimethyl sulfoxide (DMSO, Certified A.C.S) was purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Preparation of standards and quality control samples

Stock solutions of ciclopirox and chloridazon were prepared by dissolving accurately weighed standards (or equivalent amount of Penlac®) in DMSO to yield the concentrations of 1.0 mg ciclopirox free acid/mL. Two separate ciclopirox stocks were prepared for standards and quality control (QC), respectively. The 50 μg/mL ciclopirox standard, QC and the chloridazon internal standard (IS) sub-stocks were prepared by diluting stock solutions with methanol–water (1:1, v/v).

Standard solutions of ciclopirox at six concentration levels and QC solutions at three concentration levels were prepared by diluting standard sub-stock solution and QC sub-stock with *in vitro* nail penetration matrix, respectively. An internal standard working solution (ISWS) containing 100 ng/mL of chloridazon was prepared by transferring 100 μL of IS sub-stock into a 50 mL volumetric flask and bringing to volume with acetonitrile: 20% ammonium hydroxide in water (3:1, v/v).

2.3. Sample processing

To each of the K_2EDTA tubes, 4 mL of ISWS was added and the tubes were vortexed. The study samples were thawed at room temperature, and mixed thoroughly by vortexing. To a 96-deep well plate, 100 μL aliquot of STD, QC and experimental samples were added to the designated wells followed by the addition of 100 μL of ISWS from the K_2EDTA tubes (except the double blanks to which 100 μL of acetonitrile: 20% ammonium hydroxide, 3:1 (v/v) were added). The plate was vortexed briefly and 2 μL from each well were injected for LC/MS/MS analysis.

2.4. Chromatographic conditions

The chromatographic separation was performed on a Waters Atlantis® T3 column (50 mm × 2.1 mm, 5 μm) that was maintained at 40 °C. A mobile phase gradient program with solvent A (0.1% formic acid in HPLC water) and solvent B (0.1% formic acid in HPLC acetonitrile) was applied at a flow rate of 0.5 mL/min. The gradient program started with 5% B for 0.5 min followed by a linear increase in B to 70% from 0.5 to 0.8 min and held at 70% for 0.7 min (from 0.8 to 1.5 min). Mobile phase B was then increased to 100% within 0.5 min, held at 100% for another 1.0 min (from 2.0 to 3.0 min) and then reduced linearly to the initial condition (5% B) within 0.1 min. This condition was held until the end of the run. The total run time was 4.0 min. For the first 1.0 min, when the salts and impurities that cannot be retained were being eluted from the column, a valve installed between the column and mass spectrometer was switched to divert column flow to waste. At the end of 1.0 min, the valve was switched to direct flow to the mass spectrometer. After both analyte and IS peaks were recorded the valve was switched back to waste at 2.6 min and maintained in that position through the conclusion of the run at 4 min.

2.5. ESI-MS/MS conditions

An AB Sciex API 4000 linear ion TRAP quadrupole mass spectrometer (4000 Q TRAP), operated in positive electrospray ionization (ESI) mode, was used for mass detection and analysis. Multiple reaction monitoring (MRM) was used to monitor the precursor → product ion transitions of m/z 208.0 → 135.8 and