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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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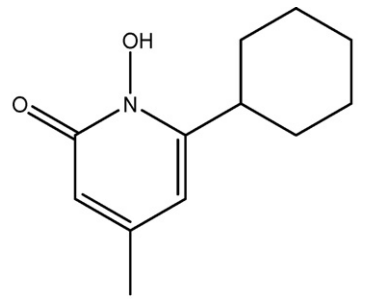

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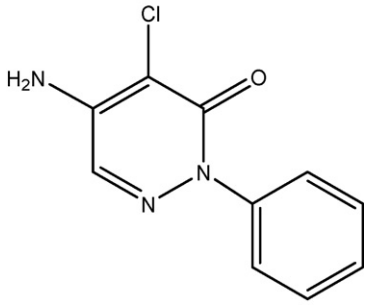

(II)

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

221.8 → 77.0 for ciclopirox and chloridazon, respectively. Dwell time for both transitions was 200 ms. The ESI ion source temperature was at 600 °C. Other optimized MS/MS parameters were: curtain gas flow: 28 psi, collision gas: 6 psi, ion spray voltage: 5500 V, ion gas 1:50 psi, ion gas 2:50 psi, entrance potential: 10 V, collision cell exit potential: 8 V for ciclopirox and 2 V for chloridazon, declustering potential: 56 V for ciclopirox and 131 V for chloridazon, and collision energy: 41 eV for ciclopirox and 57 eV for chloridazon.

2.6. In vitro nail penetration study design and sample collection

The *in vitro* nail penetration study was conducted at Cetero Research (Cary, NC, USA). The *in vitro* Franz human skin finite dose model was used for this study. The *in vitro* Franz human skin finite dose model has proven to be a valuable tool for the study of percutaneous absorption and the determination of the pharmacokinetics of topically applied drugs. The model uses human *ex vivo* cadaver or surgical skin mounted in specially designed diffusion chambers allowing the skin to be maintained at temperature and humidity that match typical *in vivo* conditions [11]. For this study, a modified chamber was used and human cadaver finger nails were mounted on a modified chamber. A finite dose (12.5 μL/cm^2) of Penlac® (Ciclopirox Solution), 8% was applied to the outer surface of the nail daily for a total of 14 days and drug absorption was measured by monitoring its rate of appearance in the reservoir solution (double deionized water, ddH_2O) bathing the inner surface of the nail. Data defining total absorption, rate of absorption, as well as nail content can be accurately determined in this model [12].

Approximately 20–30 min prior to dosing on day 8, and on study day 15, the dosed nails were gently washed using a Q-tip moistened with reagent grade ethanol, per Penlac prescribing information. Nail surfaces were then be gently wiped dry with a dry Q-tip. On days 2–7 and days 9–14, nail washes using Q-tips moistened with water were performed approximately 30 min prior to dosing on that day, daily.

At 48 h intervals, the receptor solution was removed in its entirety, replaced with fresh receptor solution, and an aliquot (approximately 1.5 mL) was frozen immediately for subsequent analysis. The analyzed ciclopirox concentration was converted to the amounts in reservoir solution by the following equation.

$$\text{Ciclopirox amount}\,(\mu\text{g/cm}^2) = \frac{\text{ciclopirox concentration}\,(\text{ng/mL}) \times \text{reservoir solution volume}\,(\text{mL})}{1000 \times \text{nail surface area dosed}\,(\text{cm}^2)}$$

Prior to the skin penetration study the stabilities of Penlac® diluted to 1 and 10 μg/mL in ddH_2O were evaluated. The recoveries of ciclopirox from the nail matrix were also investigated by spiking the *in vitro* nail matrix that was pre-bathed with inner surface of the nails with cilopirox at two concentration levels and comparing their peak area ratios of ciclopirox/IS with that in neat ddH_2O.

3. Results and discussion

3.1. Method development and optimization

In order to prevent ciclopirox from irreversibly interacting with trace dissolved metal ions and the silica gel adsorbent, the ethylenediaminetetraacetic acid dipotassium salt (K_2EDTA) containing tube was used in ciclopirox sample preparation. EDTA is a well-known strong chelator. When combined with ciclopirox, EDTA competed with ciclopirox in terms of the chelating effect and prevented ciclopirox from binding with trace metal ions and therefore, making the direct assay of ciclopirox possible. The K_2EDTA tubes are commonly used in pharmaceutical and biopharmaceutical industries for the production of plasma samples from *in vivo* animal studies. The tubes are made in different dimensions containing different amounts of spray-dried K_2EDTA. The volumes of blood collection vary dependent on the different sizes of tubes used resulting the same K_2EDTA concentration of 1.8 mg/mL, e.g. 7.2 mg/4 mL blood, 5.4 mg/3 mL blood, etc. al. The concept of using K_2EDTA tubes instead of solid bulk material was the result of two reasons. One was the K_2EDTA concentration in the plasma samples generated from the K_2EDTA tubes was widely used in bioanalytical analysis and it is well tolerated by LC/MS systems even when the plasma samples are directly injected into the system. The other was their ease of use. The pH effect was studied using 20% ammonium hydroxide aqueous solution, 0.1% formic acid aqueous solution and 5 mM ammonium acetate buffer. The basic, acidic or neutral solution was mixed with acetonitrile at a ratio of 1:3 (v/v) in the K_2EDTA tube and then, mixed with ciclopirox solution at ratio of 1:1 (v/v). It was found that the best sensitivity was achieved with alkaline sample while the sensitivities of acidified and neutral samples were beyond the satisfactory level (Fig. 2). This was due to the fact that the capability of EDTA chelation increases with pH increase [10]. It might also indicate that the chelating effect of ciclopirox with pKa of 7.2 was probably also pH dependent. The presence of organic solvent in LC/MS/MS assay samples is important as it enhances the desolvation of the sample droplet in the ion source and improves the ionization efficiency. Acetonitrile was used as organic solvent in this study and the different ratios of acetonitrile vs. 20% ammonium hydroxide aqueous solution were compared for assay sensitivity. With consideration of K_2EDTA solubility, the maximum ratio tested was 5:1 (v/v). At this ratio, the ciclopirox peak was tailed with low sensitivity. This result possibly occurred due to insufficient dissolved K_2EDTA available to compete with ciclopirox from binding. The good sensitivity and peak shape were obtained at the ratio of 3:1 (v/v) and this ratio was used for the method validation and sample analysis. The impact of LC/MS/MS mobile phases on ciclopirox peak shape and assay sensitivity was also evaluated. There was no significant difference found between the buffer (A: 5 mM ammonium acetate; B: acetonitrile) and acidic (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile)

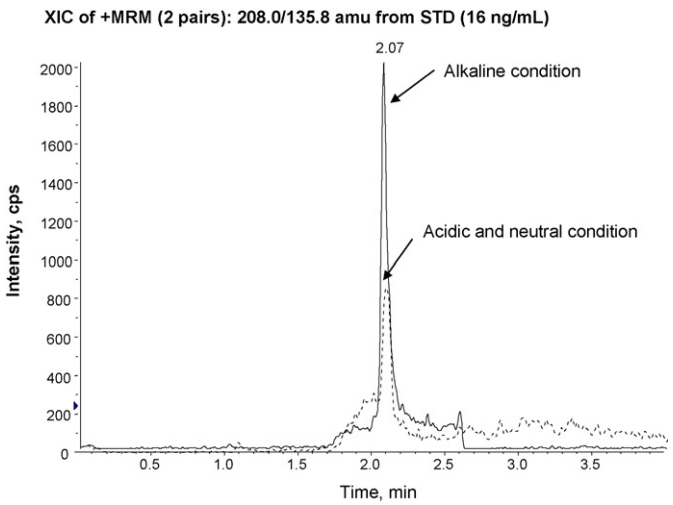

Fig. 2. Effect of alkaline sample processing condition on the sensitivity improvement of ciclopirox. Dashed line: ciclopirox standard (16 ng/mL) mixed with acetonitrile: HPLC water, 3:1 (v/v). Solid line: ciclopirox standard (16 ng/mL) mixed with acetonitrile: 20% ammonium hydroxide in water, 3:1 (v/v).

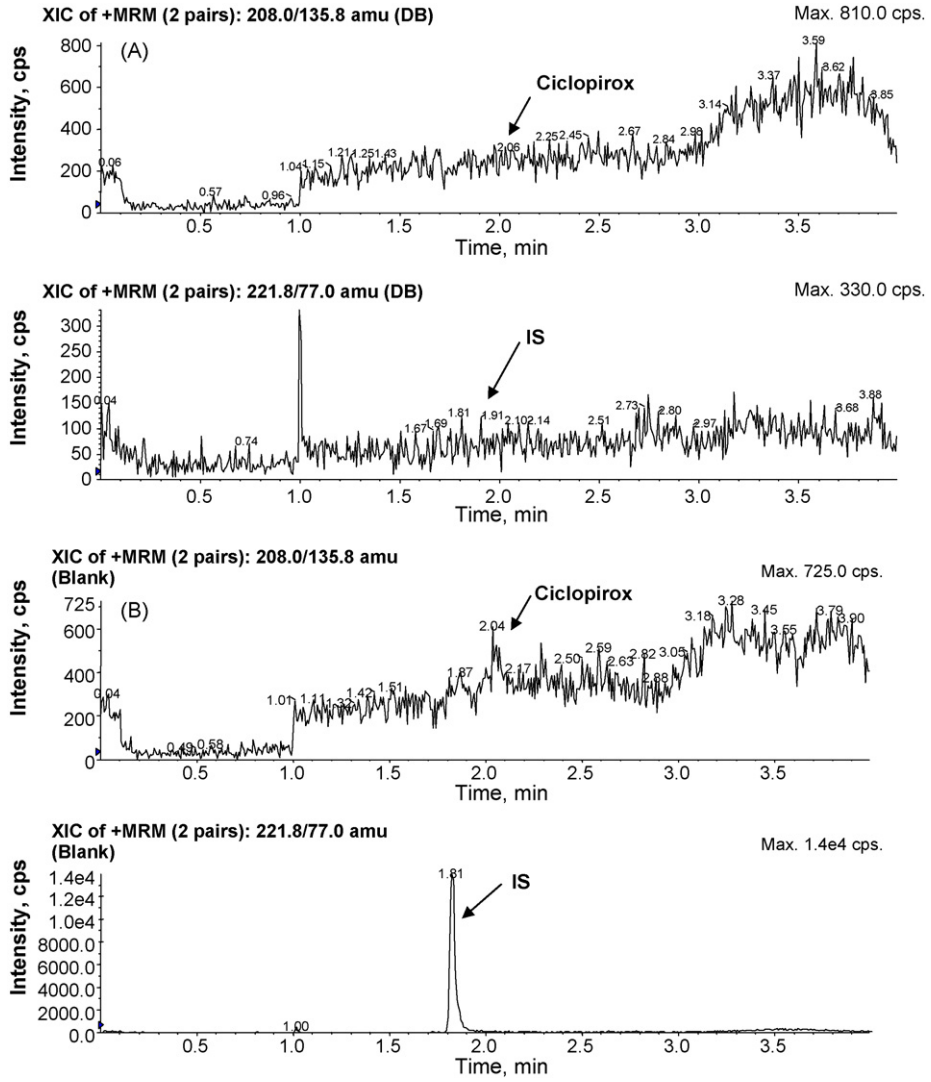

Fig. 3. Representative chromatograms of a blank matrix without IS (A), blank matrix with IS (B).

mobile phases. The acidic mobile phases were chosen for further studies.

3.2. Method validation

3.2.1. Specificity, recoveries and sensitivity

Analysis of three different blank nail matrices, double deionized water (ddH_2O), 1× phosphate buffered saline (1× PBS) and saline showed no interference peaks appeared at the retention times of either analyte or internal standard. The recoveries of ciclopirox from nail matrix (ddH_2O pre-bathed with inner surface of nail) were studied at two concentration levels. The recoveries of ciclopirox from nail matrix were 103% at 1 μg/mL and 99.0% at 10 μg/mL, respectively. These results demonstrate good specificity of the method with no matrix effect. The representative chromatograms of ddH_2O with and without internal standard are shown in Fig. 3. The assay LLOQ was studied using two different ciclopirox materials, commercially available ciclopirox and prescription Penlac. Both materials showed the detectable level of ciclopirox at 2 ng/mL. Considering the signal-to-noise (S/N) ratio of 10, the LLOQ was set at 8 ng/mL. The precision and accuracy at LLOQ determined in intra-assay ($n = 6$) were 4.56% and 110%, respectively (Table 1). The representative chromatograms of ciclopirox at LLOQ and at upper limit of the quantitation (ULOQ) are shown in Fig. 4.

Table 1
Intra-assay and inter-assay precision and accuracy.

Assay type	Nominal concentration	n	Mean calculated concentration	Precision (% CV)	Accuracy (% nominal)
Intra-assay	8.00	6	8.82	4.56	110
	40.0	6	42.6	7.42	106
	200	6	214	2.70	107
Inter-assay	8.00	21	9.01	6.57	113
	40.0	21	40.5	10.6	101
	200	21	213	8.26	106

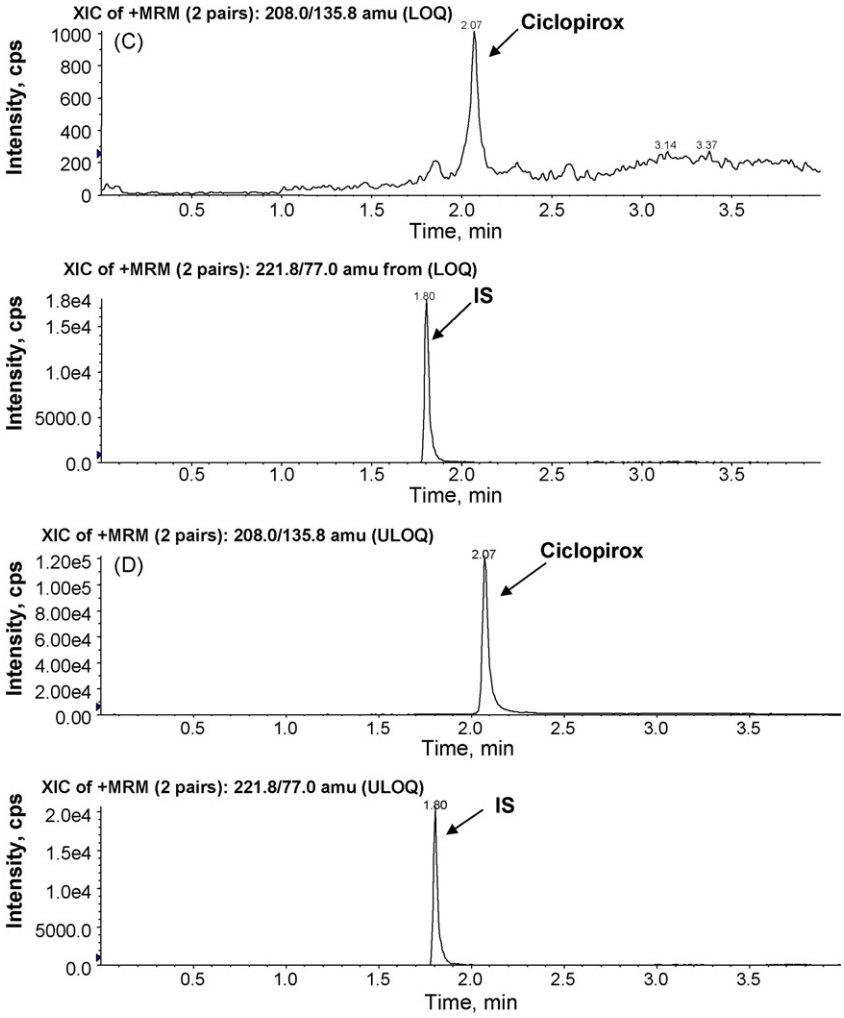

Fig. 4. Representative chromatograms of an LLOQ sample at 8.0 ng/mL (C) and an ULOQ sample at 256 ng/mL (D).

3.2.2. Precision and accuracy

The intra-assay precision and accuracy were evaluated by analyzing within the same run six replicate QC samples at each of three concentration levels. The intra-assay precision ranged from 2.70% to 7.42%, and the accuracy, expressed as percentage of nominal values, ranged from 106% to 110% (Table 1). The inter-assay precision determined by analyzing triplicate QC samples at each of three concentration levels for six sets of runs (including one intra-assay) were between 6.57% and 10.6% and inter-assay accuracy ranged from 101% to 113% (Table 1).

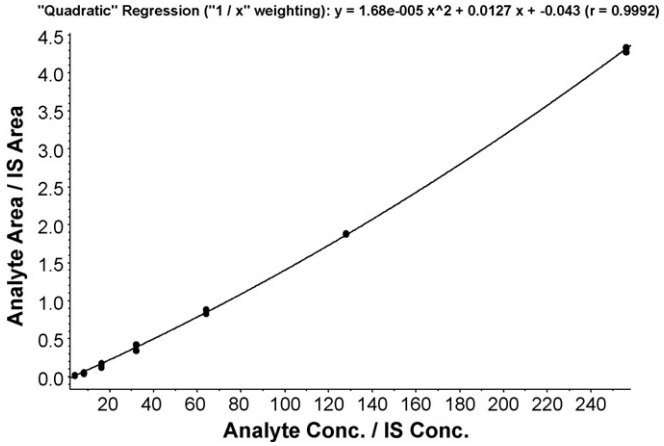

Fig. 5. A representative calibration curve.

3.2.3. Calibration reproducibility

In each of validation sessions, fresh calibration samples at six concentration levels were prepared and analyzed as described above. The calibration curves were generated using weighted

Table 2
Summary of calibration regression results.

	Concentration (ng/mL)					
	8	16	32	64	128	256
Assay no.						
1	7.84	16.2	31.7	66.4	125	257
2	7.81	16.3	33.2	61.5	129	256
3	9.39	15.1	27.5	67.1	128	256
4	9.13	14.7	28.2	67.2	129	255
5	8.22	15.2	32.8	64.3	128	256
6	9.44	13.3	31.6	63.4	130	255
Parameters						
Mean	8.64	15.1	30.8	65.0	128	256
% CV	8.89	7.27	7.79	3.54	1.34	0.294
% nominal	108	94.6	96.4	102	100	99.9
n	*6*	*6*	*6*	*6*	*6*	*6*

Table 3
Formulation stability results.

Storage length (day)	Room temperature				32 °C			
	1 μg/mL	% change	10 μg/mL	% change	1 μg/mL	% change	10 μg/mL	% change
0	0.919	–	11.6	–	0.919	–	11.6	–
1	0.983	7.0	11.7	0.86	0.969	5.4	11.6	0.0
2	0.922	0.33	11.9	2.6	0.964	4.9	12.0	3.4
3	0.914	−0.54	11.6	0.0	0.917	−0.22	11.6	0.0

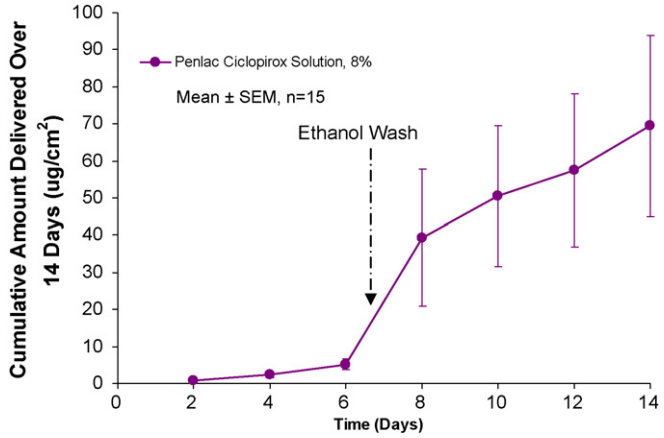

Fig. 6. The cumulative ciclopirox amounts in reservoir solution after daily 14 days of dosing.

(1/concentration) least-squares quadratic regression mode over a concentration range of 8–256 ng/mL, with correlation coefficients (r) equal to or greater than 0.9991. A representative calibration curve is shown in Fig. 5 and the calibration standard parameters representing calibration reproducibility are listed in Table 2.

3.2.4. Stability

The post-processed ciclopirox samples were found stable for at least 7 days in the autosampler at 4 °C with percentage of loss ranging from 0.95% to 1.3% for low QC (LQC) and high QC (HQC) samples tested. After three freeze/thaw cycles, ciclopirox in nail matrix demonstrated acceptable stability with percentage losses of −1.7% for LQC and −0.16% for HQC, respectively. The storage stability was evaluated by analyzing formulation samples at 1 and 10 μg/mL concentration levels. The results indicated that the formulation samples were stable for at least 3 days at room temperature and 32 °C (Table 3).

3.3. Nail penetration assay results

At 2, 4, 6, 8, 10, 12, and 15 days post-dosing, the ciclopirox concentrations in the reservoir solutions from 15 different skin donors were analyzed using the validated method. The mean cumulative amounts of ciclopirox in reservoir solution are shown in Fig. 6. The accumulated amount of ciclopirox penetrated through the nail on day 15 was 69 ± 92 μg/cm^2.

4. Conclusion

A rapid, sensitive and direct liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the determination of ciclopirox in *in vitro* nail penetration samples. The method was validated over a concentration range of 8–256 ng/mL ($r > 0.9991$). The intra- and inter-day assay accuracy (% of nominal) and precision (%CV) were, respectively, within ±113% and ≤11%. No matrix interference at the retention time of ciclopirox and internal standard, chloridazon. The validated method was successfully utilized to accurately analyze 225 experimental samples for a nail penetration study. The formulation stability was also evaluated using the validated method.

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