Development and Validation of Liquid Chromatography–Tandem Mass Spectrometry Method for Simultaneous Determination of Zinc Pyrithione and Pyrithione in Shampoos

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A simple, rapid, and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and validated for the determination of zinc pyrithione (ZnPT) and pyrithione (PT) in shampoos. The method consisted of a liquid–liquid extraction for sample preparation. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode via the positive electrospray ionization interface. A linear regression (weighted $1/x$) was used to fit calibration curves over the concentration range of 50–2000 ng/mL for both ZnPT and PT. Excellent linearity ($r^2 \geq 0.9996$) was achieved for all. The method was validated and found to be accurate (95.9–108.2% for ZnPT and 94.9–110.4% for PT), precise, and selective. Analytes in shampoos were found to be stable in the auto-sampler (6 °C for 6 h), in room temperature (for 6 h), and after three freeze–thaw cycles, and recovery of analytes was reproducible (90.8–94.6% for ZnPT and 90.2–96.3% for PT).

Keywords: Pyrithione, zinc pyrithione, LC–MS/MS, liquid–liquid extraction, shampoos

Introduction

Zinc pyrithione (ZnPT) is a coordination complex of zinc with the chemical formula $C_{10}H_8N_2O_2S_2Zn$, resulting from combination of 2-mercaptopyridine-1-oxide with zinc (II) (Figure 1). It is also known as zinc pyridinethione, bis(2-pyridylthio) zinc, 2-mercaptopyridine *N*-oxide zinc salt, and under the commercial names of Danex, De-Squaman, Zinc Omadine, and Vancide ZP, which has been widely used as a bactericide, fungicide, algicide, and even an antidandruff agent. ZnPT can transform into copper pyrithione (CuPT) by transchelation with copper ion in the laboratory and under natural conditions [1, 2]. The environmental toxicity of ZnPT or CuPT and their degradation products on microalgae, macrophytes, crustaceans, fish, sea urchin, and other organism have been studied [3–13]. ZnPT causes morphological abnormality in both killifish and zebrafish [5]. CuPT causes skeletal deformity in the fish *Fundulus heteroclitus* [9]. The toxicity of ZnPT after subchronic oral dosing has been studied in several animal species. Dogs dosed with ZnPT 6–12 mg/kg/day for 6 days show ocular lesions involving the tapetum lucidum [14, 15]. Rats fed with a diet containing 250 ppm ZnPT for 10 days showed locomotor abnormalities accompanied by muscle atrophy [16]. Rabbits dosed with ZnPT 17 mg/kg/day for 14 days developed hind-limb weakness that is associated with a dying-back neuropathy [17].

Based on its toxicity, ZnPT is allowed in a concentration of 1% for preservative purposes in cosmetic rinse-off hair care products and 2% for rinse-off antidandruff hair care products in the European Union (EU) [18]. Approximately 28% of the shampoos in circulation on the market in 2013 have been reported to contain ZnPT and ethylenediaminetetraacetic acid (EDTA) together. EDTA will chelate the zinc from ZnPT and dissociate ZnPT into anion of pyrithione (Figure 2). This decomposition is speculated to increase the absorption of pyrithione anion and hence increase concerns about resulting

Figure 1. Structure of zinc pyrithione (A) and pyrithione (B)

toxicity. ZnPT has been reported to demonstrate low penetration, whereas sodium pyrithione (NaPT) with high water solubility was shown that it can be absorbed through skin in much greater amounts [19]. As a result, NaPT was designated as a prohibited ingredient in shampoo formulation. However, the current situation has been left without any criteria with respect to the formulation of ZnPT and EDTA.

There are many analytical methods for the determination of ZnPT in shampoos, environmental, and biological matrices [20–25]. However, chromatographic techniques for ZnPT and PT determination are rare, although the use of electrochemical techniques such as polarography, voltammetry, and amperometry have been investigated [26–30]. It has been analyzed by the way of radio-labeling, derivatization with fluorescing group, or transchelation to Cu (II) complex. However a direct, simple, and simultaneous analysis method for ZnPT and pyrithione (PT) is still lacking. Therefore, we attempted to develop and validate a sensitive and specific analytical method for the simultaneous determination of ZnPT and PT in shampoos using liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Experimental

Chemicals and Reagents. Zinc pyrithione (ZnPT), pyrithione (PT), internal standard (IS), d₃-5-nitro-5'-hydroxy-indirubin-3'-oxime (d₃-AGM-130), ethylenediaminetetraacetic acid (EDTA), formic acid, and ammonium acetate were

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Mass spectrum of compound 1 showing relative abundance versus m/z . The base peak is at m/z 78.134. Other significant peaks are labeled at m/z 51.134, 110.094, 127.095, 144.168, 189.995 (circled in red), 208.049, 226.076, 266.833, and 300.063.

purchased from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA). Methanol (MeOH), chloroform, and water were purchased from Burdick and Jackson (Muskegon, MI, USA). All other chemicals and reagents were analytical grade and used without further purification.

mixed thoroughly and then diluted 5 times with distilled water. The final diluted shampoo formulations (1% ZnPT with and without 2% EDTA) were used in experiments.

The stock solutions of ZnPT, PT, and IS (d₃-AGM-130) were prepared separately by dissolving them in chloroform-MeOH (2:1, v/v) to yield a final concentration of 1 mg/mL. The stock solutions were then diluted with methanol to yield standard working solutions of 50 to 2000 ng/mL for ZnPT and PT. A working solution of the IS was prepared by diluting IS stock solution with methanol to yield a final concentration of 5 µg/mL.

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Table 1. MS/MS conditions for zinc pyrithione (ZnPT), pyrithione (PT), and IS (d_3 -AGM-130)

Parameters	ZnPT	PT	IS (d_3 -AGM-130)
Mass transition	317 → 190	128 → 110	342 → 279
Collision energy (eV)	20	15	37
Retention time (min)	0.90	0.67	0.64
Curtain gas (psi)		50	
Collision gas (psi)		5	
Source temperature (°C)		400	

These diluted working standard solutions were used to prepare the calibration curve and quality control (QC) samples.

Calibration standard and QC samples were prepared by spiking the blank shampoo with working solutions of each analyte to yield seven different concentrations over a range of 50–2000 ng/mL for ZnPT and PT. For QC samples, three concentration levels of standard samples (100, 500, and 1600 ng/mL) were prepared. All solutions were stored at -20°C .

Sample Preparation. A volume of 5 μL IS (5 $\mu\text{g/mL}$) and chloroform–MeOH (2:1, v/v) (150 μL) were added to the calibration standards, QC, and shampoo samples (50 μL), and

then the mixture were vortex-mixed for 1 min. After centrifugation at 4°C , 13,000 rpm for 5 min, the chloroform layers (50 μL) were diluted with MeOH (50 μL). The processed samples (5 μL) were analyzed by LC–MS/MS system.

Chromatographic Conditions. The liquid chromatography–mass spectrometry system consisted of an Agilent 1290 series rapid resolution LC System and triple quadrupole linear ion trap mass spectrometer (4000 Q-Trap) (AB Sciex, Foster City, CA, USA). The analytical column used for this assay was a Gemini C18 column (100 $\times$ 3.0 mm, 3 μm , Phenomenex, Torrance, CA, USA) protected by a Security Guard column (4 mm $\times$ 3 mm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of deionized water with 20 mM ammonium acetate and methanol with 0.1% formic acid (10:90, v/v). The flow rate was set at 0.35 mL/min, and the injection volume was set at 5 μL .

The Q-Trap mass spectrometer was operated in the positive ion mode using an electrospray ionization source. High purity nitrogen gas was used for the nebulizer and curtain gases. The source temperature was set at 400°C with a curtain gas flow of

Figure 5. Representative chromatograms for ZnPT (left for ZnPT; right for IS) at double blank (A), blank sample (B), LLOQ (50 ng/mL, C), and HLOQ (2000 ng/mL, D) in shampoo solution

Figure 6. Representative chromatograms for PT (left for PT; right for IS) at double blank (A), blank sample (B), LLOQ (100 ng/mL, C), and HLOQ (2000 ng/mL, D) in shampoo solution

50 L/min. The ion spray voltage was set at 5500 V, and the collision energy was 20 and 15 V for ZnPT and PT, respectively. The following multiple reaction monitoring (MRM) transitions of the respective $[M + H]^+$ ions were used to quantify ZnPT and PT: ZnPT, m/z 317 $\rightarrow$ 190; PT, m/z 128 $\rightarrow$ 110; and d_3 -AGM-130 (IS), m/z 342 $\rightarrow$ 279. The dwell time for each transition was 150 ms. Analyst software 1.6 was used to optimize the MS parameters, data acquisition, and data processing.

Validation and Sample Analysis Procedure. The validation was carried out according to the United States Food and Drug Administration (FDA) guidance for industry on bioanalytical method validation guidance [31]. Accuracy and precision were determined by quantification of the QC samples at four concentrations (LLOQ, low, mid, and high concentrations). Intra-day variability was determined by analyzing the QC samples three times using the same calibration curve. Inter-day variability was determined by analyzing the QC samples on three different days using calibration curves constructed on the respective days. Accuracy was calculated as the relative difference between calculated and nominal concentration of

the QC samples (bias, %), and precision was expressed as the relative standard deviation (RSD). The intra- and inter-day accuracy and precision should be within $\pm 15\%$ except at LLOQ, which was set at within $\pm 20\%$. The stability and recovery were evaluated at low, mid, and high concentrations ($n = 3$ at each concentration).

Results and Discussion

Liquid Chromatography–Tandem Mass Spectrometry Optimization. In this study, several conventional solvents such as methanol, acetonitrile, ethanol, and acetone were evaluated for method development. Even with 90% methanol, the peak appeared at the solvent front with tailing. Addition of 20 mM ammonium acetate to the eluent was found to eliminate this problem. The best peak shape and retention time for ZnPT and PT were observed using methanol (containing 0.1% formic acid)/water (containing 20 mM ammonium acetate) (90:10, v/v) as the eluent. Chloroform, chloroform–methanol, ethyl acetate, and methyl-tert-butyl-ether were evaluated as

extraction solvents for liquid–liquid extraction during method development. Among them, chloroform–methanol (2:1, v/v) was found to give the best extraction efficiency with less interference and faster sample preparation time.

The analytes were then separated on the C18 column within 2 min using isocratic elution. The collision energy and source temperature were carefully optimized for analytes and IS to produce the highest response in the detector. Product ion mass spectra of analytes are shown in Figures 3 and 4. Retention times, collision energy, gas pressure, and MRM transitions for the analytes and IS are shown in Table 1. All analytes formed protonated molecules of $[M + H]^+$ when the mass spectrometer was operated under positive ionization mode. Dissociation was allowed to occur completely at 400 °C, and quantification of ZnPT, PT, and IS was carried out using MRM of m/z 317 → 190, 128 → 110, and 342 → 279, respectively.

Method Validation. Calibration curves were plotted based on the response ratio of analyte to internal standard versus the concentration of analyte. The calibration curve ranged from 50 to 2000 ng/mL for ZnPT and PT and exhibited excellent linearity with $r^2 > 0.9996$ using weighting of $1/x$. Representative chromatograms of the calibration standards at the lower limits of quantitation (LLOQs) (signal-to-noise ratio, >10) are shown in Figures 5 and 6.

The intra- and inter-day accuracy as well as precision values of the method were assessed using spiked shampoo samples at concentrations of 50, 100, 500, and 1600 ng/mL for ZnPT and PT. The intra- and inter-day accuracy values (%) were in the range of 95.9–108.2% for ZnPT and 94.9–110.4% for PT. The intra- and inter-day precision values (CV, %) were in the range of 1.8–10.5% for ZnPT and 0.8–13.9% for PT (Table 2). These data have met the requirement of the FDA guidance, and the developed method was shown to be accurate and reproducible in the simultaneous analysis of ZnPT and PT.

The developed method is specific and highly selective because no coeluting peaks were found in the drug-free shampoos (Figures 5 and 6). Recovery was determined by comparing the mean peak areas of low, mid, and high QC samples (processed) with the mean peak areas of extracted blank samples spiked with solution containing analyte and IS at concentrations representing 100% recovery (unprocessed). Recovery of ZnPT in the low, mid, and high QC samples was 93.9%, 90.8%, and 94.6%, respectively, and recovery of PT was 93.4%, 90.2%, and 96.3%, respectively (Table 3). The results show that the liquid–liquid extraction method is efficient.

Stability assessments were carried out to demonstrate that ZnPT and PT were stable under typical sample storage and processing conditions. The stability experiments for shampoos were performed using QC samples ($n = 3$) at the low, mid, and high QC levels. ZnPT and PT in shampoos were stable at room temperature for 6 h, at autosampler tray (4 °C) for 6 h, and through three freeze–thaw cycles (Table 4).

Table 2. Intra- and inter-day accuracy and precision of ZnPT and PT in LLOQ and QC samples

	Concentration (ng/mL)	Intra-day ($n = 3$)		Inter-day ($n = 3$)	
		Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)
ZnPT	50	96.4	10.5	101.2	10.1
	100	101.9	3.7	108.2	2.4
	500	104.7	2.5	95.9	2.0
	1600	103.4	2.3	106.3	1.8
PT	50	107.3	13.9	110.4	12.5
	100	94.9	4.6	103.1	6.7
	500	102.4	0.8	97.3	2.3
	1600	107.6	3.4	105.5	1.4

Table 3. Recovery of ZnPT and PT in QC samples

	QC samples (ng/mL)	Recovery ($n = 3$)	
		Mean (%)	CV (%)
ZnPT	100	93.9	12.0
	500	90.8	4.3
	1600	94.6	8.2
PT	100	93.4	13.3
	500	90.2	4.1
	1600	96.3	4.5

Table 4. Stability of ZnPT and PT in QC samples ($n = 3$)

	QC samples (ng/mL)	Autosampler (4 °C, 6 h)		RT (6 h)		Freeze–thaw (–20 °C, 3 cycles)	
		Mean (%)	CV (%)	Mean (%)	CV (%)	Mean (%)	CV (%)
ZnPT	100	104.8	5.9	97.7	7.6	102.3	0.6
	500	102.7	3.5	94.4	3.5	105.3	2.9
	1600	103.6	1.4	104.4	2.3	96.5	1.7
PT	100	102.3	8.0	105.2	3.8	97.5	6.0
	500	106.9	4.9	98.8	5.1	93.7	3.8
	1600	96.4	2.6	97.3	2.0	103.4	1.8

Table 5. Analysis results of ZnPT and PT in 1% of zinc pyrithione shampoo samples

	Without EDTA	With EDTA
ZnPT (μg/mL)	9790	5820
PT (μg/mL)	1	1730

Application of Methods. The validated LC–MS/MS method was successfully applied to the ZnPT shampoo samples with and without EDTA. The results are given in Table 5.

Conclusions

A rapid, precise, and accurate liquid chromatography–tandem mass spectrometry method was developed and validated for the simultaneous determination of ZnPT and PT in shampoos. The calibration curve showed goodness of fit over varying concentrations that ranged from 50 to 2000 ng/mL for ZnPT and PT. Intra- and inter-day accuracy and precision for calibration standards and QC samples met the suggested industry and regulatory acceptance criteria. ZnPT and PT were stable in shampoos and extracts under the storage and test conditions used for this study. This liquid chromatography tandem–mass spectrometry method is very sensitive, selective, accurate, and reproducible for the determination of ZnPT and PT concentration in shampoos.

The simultaneous assay of ZnPT and PT from the ZnPT shampoos with and without EDTA has been applied successfully for the formulation analysis. There might be increased risk of absorbed PT toxicity of ZnPT containing shampoo by the addition of EDTA due to partial dissociation of ZnPT into PT. Therefore, this developed method should be adopted for the potential risk assessment of dissociated PT from the ZnPT and EDTA containing shampoos.

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