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UA-IL-DLLME-LC-MS/MS Method for the Detection of Chlorfenapyr and Its Metabolites

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Abstract

This study established an ultrasound-assisted ionic liquid–dispersive liquid–liquid microextraction combined with liquid chromatography–tandem mass spectrometry (UA-IL-DLLME-LC-MS/MS) method for the detection of chlorfenapyr and its toxic metabolite tralopyril in blood samples. Chlorfenapyr is a broad-spectrum pyrrole insecticide that is metabolized into tralopyril upon entering the organism, disrupting oxidative phosphorylation and leading to highly lethal poisoning. Compared to the conventional liquid-liquid extraction (LLE) method, which is complex and time-consuming, the proposed approach offers advantages such as low organic solvent consumption, rapid extraction, and high efficiency. The experimental conditions were optimized, and the method was validated for specificity, linear range, limit of detection, limit of quantification, accuracy, and precision. The results demonstrate that this method is simple, efficient, and highly sensitive, making it suitable for clinical and forensic toxicological analyses.

Keywords: chlorfenapyr, tralopyril, dispersive liquid-liquid microextraction, LC-MS/MS, forensic toxicology

1. Introduction

Chlorfenapyr (bromofenapyr), is a novel broad-spectrum pyrrole insecticide developed by the American Cyanamid Company in the 1980s. Chlorfenapyr itself exhibits weaker toxicity, but after entering the organism, it undergoes oxidative removal of the N-ethoxymethyl group to form the toxic metabolite tralopyril [1], which disrupts the oxidative phosphorylation process, inhibits energy production in insects, and thereby exerts its toxic effect [2–3].

Although chlorfenapyr is classified as moderately hazardous by the World Health Organization (WHO) toxicity classification (USEPA), case reports indicate that chlorfenapyr entering the body through oral ingestion, inhalation, or dermal contact consistently manifests as highly lethal poisoning. In humans, chlorfenapyr poisoning presents unique clinical features: early symptoms are inconspicuous and may include nausea, fatigue, fever, rhabdomyolysis, and severe delayed neurological damage. As the condition progresses, cardiac arrest occurs approximately 4–21 days following ATP depletion, ultimately resulting in unresponsive fatal outcomes with an extremely high case fatality rate [4–9]. In recent years, reported cases of chlorfenapyr poisoning have increased annually due to its widespread use.

Currently, liquid-liquid extraction (LLE) is commonly employed for pretreatment of chlorfenapyr and tralopyril in biological samples. Although reported methods can meet analytical requirements, the LLE procedure is complex, time-consuming, and requires large volumes of organic solvents. Conversely, dispersive liquid-liquid microextraction (DLLME) enhances the contact surface between aqueous phases and extractants by adding dispersants, enabling rapid transfer of target compounds between sample solutions and extractants. This method offers advantages including low organic solvent consumption, short extraction time, high extraction efficiency, and high enrichment rates [10]. Meanwhile, ionic liquids (ILs) as novel extractants have been applied in food, water, and biological sample pretreatment due to their low vapor pressure, thermal stability, and environmental friendliness [11–13]. Therefore, this study established an ultrasound-assisted ionic liquid–dispersive liquid–liquid microextraction coupled with liquid chromatography–tandem mass spectrometry (UA-IL-DLLME-LC-MS/MS) method for detecting chlorfenapyr and tralopyril in blood, providing reference value for detecting such poisoning incidents.

2. Materials and Methods

2.1 Instruments and Reagents

HPLC (Essentia LC-16)-8045MS/MS liquid chromatography-tandem mass spectrometer (Shimadzu Corporation, Japan); VORTEX-GENIE2 vortex mixer (Scientific Industries, USA); pipettes (Eppendorf, Germany); Milli-Q ultrapure water system (Millipore, USA); Neofuge 15R high-speed refrigerated centrifuge (LiShen Scientific Instrument Co., Ltd., Shanghai); SB-5200DTD ultrasonic cleaner (Xinzhi Biotechnology Co., Ltd., Ningbo).

Chlorfenapyr, tralopyril, and warfarin-D5 standards (99.9%, Tianjin Alta Technology Co., Ltd.); 1-butyl-3-methylimidazolium-hexafluorophosphate([Bmim][PF6],99.0%),1-hexyl-3-methylimidazolium-hexafluorophosphate([Hmim][PF6],99.0%),1-octyl-3-methylimidazolium-hexafluorophosphate ([Omim][PF6], 99.0%) (all purchased from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences); acetonitrile (HPLC grade, Sigma-Aldrich, USA); formic acid (chromatographic grade, Aladdin, Shanghai); ultrapure water for experiments; acetone (analytical grade, Tianjin Comio Chemical Reagents Co., Ltd.).

2.2 Sample Preparation

Pipette 100 μL of blood sample and place it in a 1.5 mLEP tube, first add 40 μL of warfarin D5 (50 ng/mL) internal standard solution, then add 60 μL of [Bmim] [PF6] extractant, then add 800 μL of ultrapure water and 40 μL of 4% NaCL solution, vortex for 30 s, add 250 μL of acetone, continue vortexing for 30 s, cold bath ultrasound for 15 min, and centrifuge in a refrigerated high-speed centrifuge at 4 °C and 6000 r/min for 10 min. Use a micropipette to aspirate 10 μL of the lower ionic liquid and transfer it to another 1.5 mLEP tube, add 90 μL of methanol and filter using a 0.22 μm Nylon filter.

2.3 Chromatographic and Mass Spectrometry Conditions

Chromatographic ConditionsColumn: ZORBAX Hilic Plus C18 (4.6 mm × 100 mm × 3.5 μm); Flow rate: 0.6 mL/min; Pure water (A), acetonitrile (B); Injection volume: 5 μL. The gradient elution program is: 0–1.0 min, 10–90% B; 1.0–3.0 min, 90–100% B; 3.0–4.0 min, 100-10% B; 4.0–5.0 min, 10% B. Mass Spectrometry Conditions: using MRM (Multiple Reaction Monitoring) mode; The temperature of the desolvation line (DL) is 250 °C; The temperature of the atomization drying gas (TEM) is 300 °C; The parameters for the MRM of chlorfenapyr, tralopyril and warfarin D5 are shown in Table 1.

Table 1 Specific MRM parameters of chlorfenapyr and tralopyril

	ESI	Rt (min)	Precursor ion (m/z)	Product ion (m/z)	Q1/V	CE/V	Q3/V
Chlorfenapyr	-	1.18min	349.2#	80.1*/268.1	13/15	33/28	19/21
Tralopyril	-	1.83min	347	131.2*/79	11/11	42/36	23/19
IS	-	1.78min	290.2	161.1*/255.15	13/13	19/22	16/26

*Quantifier ion

#Chlorfenapyr is cleaved within a potential source within the ion source, resulting in unstable detection of [MH] (m/z 408) or [M-H]− (m/z 406) ions. In negative multiple reaction monitoring (MRM) mode, chlorfenapyr was easily cleaved into compounds with an m/z value of 349.2

3. Results

3.1 Method Specificity

Six blank blood samples from different sources were spiked with chlorfenapyr, tralopyril, and the internal standard warfarin-D5. After sample processing, the chromatographic specificity was satisfactory, with retention times of 1.18 min, 1.83 min, and 1.78 min for chlorfenapyr, tralopyril, and warfarin-D5, respectively (Figure 1).

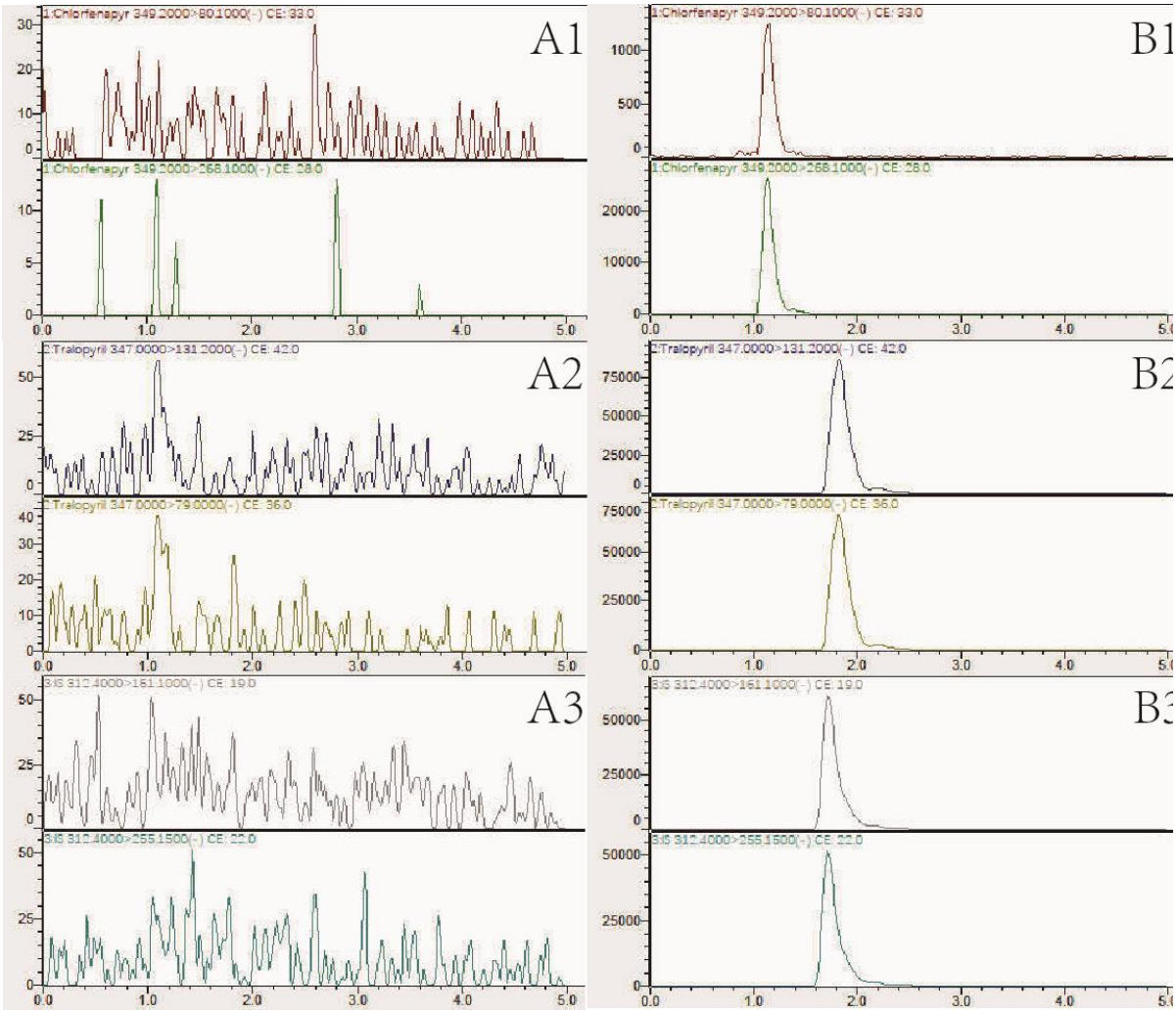

Figure 1. MRM chromatograms of blank (A1-3) and blood samples supplemented with chlorfenapyr (B1), tralopyril (B2), and warfarin-D5 (B3)

3.2 Calibration Curve, Limit of Detection (LOD), and Limit of Quantification (LOQ)

Blank blood samples (100 μL) were spiked with gradient concentrations of chlorfenapyr and tralopyril standard solutions to prepare calibration curves ranging from 1 ng/mL to 1000 ng/mL for both analytes. The processed samples were analyzed by LC-MS/MS to establish the linear ranges of the target compounds in blood (Table 2).

Table 2. Standard calibration curves for chlorfenapyr and its metabolites in blood

Analytes	Linear equation	r	Linearity range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
Chlorfenapyr	Y = 0.021X + 0.0078	0.998	1–1000	0.1	1
Tralopyril	Y= 0.013X+ 0.0043	0.996	1–1000	0.1	1

The signal-to-noise ratios (SNRs) of chlorfenapyr and tralopyril were determined to establish their LODs and LOQs. The LOD was defined as the concentration corresponding to SNR ≥ 3, while the lower limit of quantification (LLOQ) was defined as SNR ≥ 10. Sensitivity was evaluated based on the LOD and LLOQ.

3.3 Accuracy and Precision

Standard solutions of chlorfenapyr and tralopyril were separately spiked into blank blood samples to prepare low-, medium-, and high-concentration fortified samples. The concentrations were as follows: chlorfenapyr at 10 ng/mL (low), 100 ng/mL (medium), and 1000 ng/mL (high); tralopyril at 10 ng/mL (low), 100 ng/mL (medium), and 1000 ng/mL (high). Processed by the above method, six replicates were prepared for each concentration. The

samples were analyzed at three time points (morning, noon, and evening) within the same day to determine the intra-day accuracy and precision. Additionally, the samples were analyzed in triplicate at noon over three consecutive days to determine the inter-day accuracy and precision.The relative recovery (rel.R%) was calculated from the processed data to evaluate accuracy, while the coefficient of variation (CV%) was used to assess precision. The accuracy ranges for chlorfenapyr and tralopyril in blood were 93.45%–101.54% and 95.41%–102.78%, respectively. The intra-day and inter-day precision, expressed as relative standard deviation (RSD), ranged from 3.46% to 13.51% for chlorfenapyr and from 4.56% to 12.18% for tralopyril. These results meet the requirements for sample analysis.

3.4 Extraction Recovery and Matrix Effect

Follow the sample preparation procedures described above, pre-extraction and post-extraction spiked samples at low, medium, and high concentrations were prepared. The extraction recovery of the analytes was calculated based on the processed data. Standard solutions at low, medium, and high concentrations were directly prepared and analyzed by the instrument. The peak areas of these standard solutions were compared with those of the post-extraction spiked samples to determine the matrix effect. The extraction recovery of chlorfenapyr ranged from 91.42% to 98.36%, with a matrix effect of 93.41% to 103.92%. For tralopyril, the extraction recovery ranged from 93.93% to 97.10%, with a matrix effect of 97.57% to 105.14%. These results meet the requirements for sample analysis.

3.5 Stability

Stability refers to the stability of the analyte under specified matrix, storage conditions, and time intervals. In this experiment, the target analytes must remain stable from sample collection until completion of analysis. This study evaluated freeze-thaw stability and room-temperature stability.

Chlorfenapyr and tralopyril standard solutions were spiked into blank blood to prepare low-, medium-, and high-concentration samples in three batches. The first batch was analyzed immediately after preparation. The second batch underwent three freeze-thaw cycles (-20°C to room temperature) before analysis. The third batch was kept at room temperature for 24 h before analysis. The results showed that the relative deviations for both chlorfenapyr and tralopyril were less than 15%, meeting the requirements for sample analysis.

4. Conclusion

This study successfully established detection methods for chlorfenapyr and tralopyril. The sample preparation method for biological specimens (blood) and the instrumental analysis method developed in this experiment fully comply with the qualitative and quantitative analysis requirements specified in the U.S. Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance. The developed methods demonstrate significant advantages, including simplicity, high efficiency, superior sensitivity, excellent accuracy, and strong specificity, thereby providing a reliable experimental basis for clinical applications and forensic identification in relevant cases.

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