Ultrasound-assisted micellar cleanup coupled with large-volume-injection enrichment for the analysis of polar drugs in blood and zebrafish samples

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ABSTRACT
A novel ultrasound-assisted micellar cleanup strategy (UAMC) coupled with large volume injection (LVI) high performance liquid chromatography (HPLC) method was proposed and successfully applied to the analysis of cefathiamidine in complex biological samples such as whole blood, plasma, serum and even zebrafish, a challenging positive real sample. Based on the micelle-biomacromolecule interaction, the phase-separation feature of surfactant micelles and ultrasound cavitation, UAMC possessed an impressive matrix cleanup capability and could rapidly reach distribution equilibrium (approximately 2 min), which enabled simultaneous sample cleanup and analyte extraction within 8 min. Due to the high cleanup efficiency of UAMC, large volume of pretreated samples could be injected for analysis without peak broadening, impurity interference and column degradation. Thus, online analyte enrichment could be automatically performed to significantly improve method sensitivity by the column-switching LVI-HPLC system, a commercial HPLC system with small modifications. The UAMC-LVI-HPLC method creatively integrated sample cleanup, analyte extraction and on-column enrichment into simple operation. In addition, the UAMC-LVI-HPLC method enabled non-matrix-matched analysis of cefathiamidine in complex biological samples. This feature was helpful to address the problems caused by conventional matrix-matched or internal standard calibration methods, such as matrix bias, increased workload, limited availability of suitable blank matrices and the use of expensive internal standards. The method had low limits of detections (e.g., 0.0051 mg/L and 0.038 μg/g), wide linear ranges (0.030–100 mg/L and 0.15–489 μg/g), good linear correlation (R² = 0.9999), satisfactory accuracy (97.6±10.9%) and excellent intra- and interday precision (0.5–4.9%). Thus, UAMC-LVI-HPLC is expected to be a promising candidate for bioanalysis in therapeutic drug monitoring or pharmacokinetic and toxicology studies in the future.

1. Introduction

Antibiotic treatment is common but remains a challenge in clinical practice, especially in critical care medicine. In patients with altered metabolism (e.g., children, the elderly, pregnant women, and the critically ill), standard antibiotic dosing might lead to a high risk of inadequate exposure or dose-related adverse effects [1]. Safe and effective treatments are based on the knowledge of toxicity and the concentration of antibiotics in biological samples. Thus, there are increasing demands for toxicology studies and therapeutic drug monitoring (TDM), and efforts have been made to develop fast, sensitive, precise, robust, simple and cost-effective analytical methods for the quantification of antibiotics in biological samples involving whole blood, plasma, serum [2] and even zebrafish, a popular model organism in toxicology studies [3]. However, due to serious interference from complex sample matrices and trace levels of analytes, tedious and labor-consuming sample preparation processes prior to chromatographic analysis are usually required, and hence it is crucial to develop efficient sample preparation methods with good performance in matrix cleanup and analyte enrichment [4].

Sample cleanup is an important issue in pharmaceutical bioanalysis, as matrix components usually hinder the process of analyte extraction [5] and interfere with quantification [6]. For conventional solid-phase extraction and liquid-phase extraction, biomacromolecules easily

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cause intractable problems including capillary clogging, irreversible or competitive absorption, degradation of sorbent materials, emulsification and loss of extraction solvent [7–9]. Thus, removal of biomacromolecules such as protein precipitation, is usually indispensable especially when dealing with complex samples containing high proteins. In addition, co-extracted matrix components might lead to a change in the intensity of analytical signals, and consequently modified calibration procedures such as matrix-matched calibration and internal standard calibration should be introduced to reduce this systematic error [10]. Unfortunately, these calibration techniques still have many limitations, such as the increased workload and time, and the lack of certified reference materials, blank matched matrices or suitable internal standards [11].

Efficient enrichment for highly sensitive analysis is another important issue, because the concentrations of analytes are at trace levels for pharmacokinetic and toxicology studies. The enrichment of analytes seems more crucial for complicated samples, as the conventional cleanup processes such as protein precipitation and solvent-based exhaustive extraction usually reduce sample dilution due to the use of a large volume of solvents [12]. Therefore, subsequent preclean-up steps are often required to meet the sensitivity requirement of chromatographic analysis. Although evaporation [13], liquid-phase micro-extraction [14–16] and solid-phase microextraction [17,18] are widely used methods, they might lead to time-consuming multiple-step procedures and possible analyte loss. Therefore, it is of great significance to develop a simple, fast and economical method that is capable of efficient sample cleanup and enrichment for the analysis of trace targets in complicated samples without matrix-matched calibration or internal standard calibration. However, it is still challenging, especially for the direct extraction and enrichment of polar analytes from aqueous-rich complex biomacromolecules [19–21].

Our recent studies show that surfactant micelles could be used for efficient sample cleanup (e.g., milk samples [22]) due to their capability of entrapping biomacromolecules into their hydrophobic core, enabling non-matrix-matched analysis without using internal standards [23]. Although the proposed method has not been applied to the complicated solid or semi-solid biological samples such as zebrafish, it still exhibits good potential for biomacromolecules removal. The efficiency of this method may be further improved by ultrasound, as many ultrasound-assisted extraction techniques possess the features of improved micelle dispersion [24], quick phase separation [25] and enhanced mass transfer [26]. Moreover, the mechanical, thermal and chemical effects of cavitation induced by ultrasonic waves could modify the structure of proteins [27], which might facilitate the interaction between micelles and matrix components. Thus, the combination of ultrasound and micelles, termed ultrasound-assisted micellar cleanup (UAMC), has great potential for synchronous analyte extraction and cleanup of complicated biomacromolecules such as blood and zebrafish samples.

One of the simplest enrichment approaches for chromatographic analysis is the large volume injection (LVI) technique, which enables online automated enrichment process. For high performance liquid chromatography (HPLC), LVI is based on the injection of a sample volume greater than 100 μL onto an analytical HPLC column with conventional dimensions [28], realizing analyte enrichment by on-column focusing [29]. LVI has the advantages of simplicity, high sensitivity, easy automation, compatibility of online analysis and reduction in labor, extraction materials or analyte loss [30]. However, LVI has to introduce large amounts of sample matrices and strong solvents (e.g., organic solvents for reversed-phase HPLC) into an analytical column, and this feature may result in some limitations such as matrix effect, column contamination or clogging [31,32], peak broadening and decrease in column performance [33]. These drawbacks limit the application of LVI to complex sample analysis, requiring thorough sample cleanup prior to injection. In this regard, the abovementioned UAMC methods could avoid using organic solvents (i.e., strong solvents) and efficiently eliminate biomacromolecules, which should be perfectly compatible with LVI techniques for reversed-phase HPLC.

Therefore, the coupling of UAMC with LVI potentially provides an attractive way to address the abovementioned issues of sample cleanup and analyte enrichment. In this work, we developed a simple, fast, reliable, sensitive and cost-effective UAMC-LVI-HPLC method for the analysis of polar drugs in blood, plasma, serum and zebrafish samples, attempting to provide a useful tool for TDM, pharmacokinetic and toxicology studies. Key parameters of UAMC and column-switching procedures of LVI were systematically investigated to obtain optimal conditions and discover the relationship between sample cleanup efficiency and the tolerance of LVI-HPLC. Cefathiamidine, a first-generation cephalosporin, is one of the most frequently prescribed antimicrobial drugs in Chinese hospitals [34]. Due to augmented renal clearance (ARC) or severe renal impairment (RI), standard dosing might lead to underexposure or overdose in children and critically ill patients [35,36], resulting in failed treatments and adverse reactions. TDM and toxicology studies of cefathiamidine are essential for successful clinical treatment, and hence the method developed in this work was applied to the analysis of cefathiamidine in real positive samples.

2. Material and methods

2.1. Chemicals and reagents

Cefathiamidine (greater than97%) was purchased from Meilunbio (Dalian, China). Triton X-114 (TX-114, biological reagent) was obtained from Solarbio (Beijing, China). Sodium chloride (99.8%, guaranteed reagent) and formic acid (FA, 88%, analytical reagent) were obtained from Aladdin Inc. (Shanghai, China). Dibasic sodium phosphate (99.0%, analytical reagent) and citric acid (99.5%, analytical reagent) were obtained from Damao Chemical Reagent Factory (Tianjin, China). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Isopropanol (HPLC grade) was purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China). All aqueous solutions were prepared and diluted with ultrapure water (18.2 MΩ at 25 °C) and stored at 4 °C. To acquire a homogeneous solution, a 30%TX-114 (w/w) solution was kept at 4 °C for more than 24 h.

2.2. Apparatus

The analytical HPLC system (LC-20A, Shimadzu Co., Kyoto, Japan) consisted of an LC-20AD pump, a CTO-20A column oven, an SPD-M20A photodiode array detector (PDA), a CBM-20A system controller, and an acquisition data software (LC Solution). Separation was performed by an Inertsil ODS-3 column (250 mm × 4.6 mm ID, 5 μm, GL Sciences, Japan) with an Anpel C18 precolumn (10 mm × 4.6 mm ID, 5 μm, Shanghai, China).

The configuration of the large volume injection device is based on column switching techniques. The device consisted of an LC-20AT pump, an SIL-20A autosampler, an LVI-precolumn and a six-port valve (FCV-12AH, Shimadzu). The autosampler was fixed with a 1000-μL injection loop, and a Scienhome C18 precolumn (13 mm × 7.8 mm ID, 10 μm, Tianjin, China) was employed for LVI enrichment. The six-port valve was used to connect the LVI device to the HPLC system.

Turbidity was measured using a WZG-205 turbidity meter (Shanghai Xinrui Instrument Co.,Ltd, Shanghai, China). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Molsheim, France). An ultrasound cleaner (KQ-500DB) was obtained from Kunshan Xinrui Instrument Co., Ltd., Kunshan, China. A frozen tissue grinder (LIUKYM-II) and stainless-steel beads (3 mm) were obtained from Guangzhou Luka sequencing instrument Co. Ltd (Guangzhou, China).
2.3. UAMC procedure

In brief, 300 μL of the homogenized sample was thoroughly mixed with 1110 μL pretreated solution containing TX-114 (5.4%, w/w), NaCl (3.8%, w/w) and formic acid (3.6%, v/v) in a 2-mL Eppendorf tube. The mixture was kept in an ultrasound bath for 8 min. The ultrasonic power was set at 200 W with a frequency of 40 kHz, and the temperature was maintained at 35 °C. Centrifugation at 13000 rpm for 3 min was performed to achieve clear phase separation. After that, the supernatant was transferred to a clean Eppendorf tube and filtered through a 0.22 μm filter for further analysis. The non-matrix-matched calibration linear curves were prepared by using 300 μL aqueous working solutions instead of spiked samples in sample preparation. For turbidity measurement, 400 μL of the filtered supernatant was diluted with ultrapure water to 14.5 mL in a detection cell.

Before homogenization, zebrafish were washed ten times with tap water and later ten times with ultrapure water. The homogenate was prepared as follows. Zebrafish were frozen at −40 °C for 20 min, and then ground at −35 °C for 3 min (one zebrafish per tube with four stainless-steel beads). After adding water to the tube (400 mg water per 100 mg of the wet weight of the zebrafish), the mixture was ground again for 3 min, and kept in a clean Eppendorf tube before further UAMC procedure.

2.4. Experimental design and statistical analysis

A single factor experiment was performed to optimize the cleanup performance of UAMC by measuring the turbidity of the supernatant. The concentrations of TX – 114 (%), NaCl (%) and formic acid (%) were fully investigated under different conditions for optimization, and the experimental schemes are shown in Table S1.

A step-by-step single factor experiment was used for preliminary optimization of the extraction efficiency in the UAMC process. To study the influence of variables on the extraction efficiency, one variable was changed and optimized at a time based on the extraction recovery and enrichment factor, while the others were kept constant as described in Table S2. Then according to the results of the single factor experiment, a Box-Behnken design was applied to further investigate the optimal conditions and the interactions between key parameters using Design Expert version 10.0 (Stat-Ease, Inc., Minneapolis, USA). Key parameters were studied at three different levels which were coded as −1, 0, 1 (Table 1). The BBD design matrix was designed with Design Expert software. A quadratic polynomial equation was fitted based on the experimental results to demonstrate the relationship between the response and variables:

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_i^2 X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \]

where \( Y \) is the predicted response; \( k \) is the number of independent variables; \( X_i \) and \( X_j \) are the coded variables; \( \beta_0, \beta_i, \beta_{ij} \) are the constant coefficient, the linear coefficients, the quadratic coefficients and cross coefficients, respectively.

2.5. LVI-HPLC conditions

A schematic of the automated LVI-HPLC procedure is shown in Fig. 1B. The pump of LVI device (Pump 1) was used for sample loading and LVI-precolumn regeneration (i.e., washing and equilibrium), and the mobile phase from this pump consisted of acetonitrile (A), water (B), methanol (C) and isopropanol (D). The HPLC was equipped with an isocratic pump (pump 2), and mobile phase consisted of acetonitrile and an aqueous solution containing 19.4 mM of dibasic sodium phosphate and 6.7 mM citric acid. The volume ratio of acetonitrile to the aqueous solution was 15:85. The flow rate was 1 mL/min. The injection volume was 500 μL, and the detection wavelength was set at 254 nm. Chromatographic separation was performed at room temperature.

The column-switching LVI procedure went through four stages: enrichment, elution, washing and equilibrium. The gradient profiles of pump 1 and valve position of the LVI device in each stage are listed in Table 2. In the first 2.5 min, 500-μL supernatant obtained by UAMC was loaded, and the analyte was retained by the LVI-precolumn using 5% acetonitrile in the mobile phase. Then, after the valve was switched from position 1 to position 2 at 2.5 min, the analyte was eluted from the LVI-precolumn to the analytical column within 1 min, and the valve was subsequently switched back to position 1. In the next stage, methanol and isopropanol were used to wash the LVI-precolumn. Finally, the gradients of pump 1 returned to the initial conditions for the equilibrium of the LVI-precolumn. During the washing and equilibrium stage, chromatographic analysis was synchronously performed and completed by the HPLC system. The whole LVI-HPLC procedure was automated and controlled by LC Solution software.

2.6. Method validation

The sensitivity, linearity, accuracy and precision were validated for the UAMC-LVI-HPLC method according to the ICH guidelines [37]. Spiked samples were prepared by adding a known quantity of the working solution into a blank matrix (after homogenization). Sensitivity was evaluated using blank samples obtained from six individuals. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on signal-to-noise ratios of 3 and 10, respectively. Accuracy and precision were determined by analyzing the QC sample at four spiked levels: 0.03, 0.09, 30, 80 mg/L for whole blood, plasma and serum, and 0.15, 0.45, 150, 400 μg/g for zebrafish. For intraday variability assessment, six replicates at each QC concentration level were determined within a day, whereas three replicates per day were analyzed for three different days to evaluate interday accuracy and precision. The mean percentage recovery and relative standard deviation (RSD, %) were used to assess accuracy and precision, respectively.

2.7. Real sample analysis

Three-month old zebrafish were purchased from Shanghai FishBio Co., Ltd, and exposed to cefathiamidine solutions for 24 h. Three concentrations (0.28 mM, 1.0 mM and 2.0 mM) were used, and the solutions were prepared and diluted with breeding water [3]. Blank controls were used to evaluate background and interference by culturing zebrafish in breeding water for 24 h. Then these zebrafish were examined after being washed and homogenized.

3. Results and discussion

3.1. Proof-of-concept

The schematic of UAMC-LVI-HPLC is shown in Fig. 1, which is presented by two consecutive steps. The first step is the process of UAMC, which is performed for synchronous complex-sample cleanup and analyte extraction. As shown in Fig. 1A, the homogenized sample and the pretreated solution were completely mixed up, and then the mixture was kept in an ultrasound bath for analyte extraction and phase separation. Micelles and micelle-biomacromolecule complexes gathered to form a bottom phase, while polar analytes remained in the upper aqueous solution. In the second step, a large volume of the obtained clean...
The possible mechanism of UAMC is presented as follows. The principle of the two-phase separation process is similar to that of cloud point extraction (CPE) [38]. However, unlike CPE, UAMC uses the surfactant-rich bottom phase here for matrix removal instead of analyte extraction. The efficient matrix cleanup is based on the fact that biomacromolecules in sample matrices can interact with the hydrophobic core of surfactant micelles [39], forming micelle-biomacromolecule complexes (Fig. 1A). The aggregation of micelles leads to the formation of surfactant-rich phase, and micelle-biomacromolecule complexes are trapped in this bottom phase. Meanwhile, as polar analytes are inclined to be retained in an aqueous medium, they are extracted into the aqueous supernatant. Consequently, by separating the surfactant-rich phase from the aqueous solution, synchronous complex-sample cleanup and analyte extraction can be easily achieved as shown in Fig. 1A. High-intensity ultrasound is used to facilitate phase separation [40] and sample cleanup [41].

3.2. Sample cleanup efficiency

3.2.1. Turbidity as an indicator for evaluating cleanup efficiency

Generally, the cleanup efficiency of sample preparation methods is roughly evaluated by observing experimental phenomena with the naked eye, including colors and cleanness of solutions. However, the observation is empirical and sometimes unreliable. As confirmed in Fig. 2, there was no observable pressure change of a precolumn after three injections of the supernatant with a dark color (pretreated sample a), while the supernatant with a light color (pretreated sample d) caused an obvious pressure increase after the second injection. This observation failed to accurately evaluate the cleanup degree and predict the pressure increase of precolumn, which was mainly caused by the particle matrices but not the colored components in the supernatant.
To address the difficulty in measuring the cleanup performance, we attempted to use turbidity as an indicator to quantitatively evaluate the cleanup efficiency. Particle interferences, especially the biomacromolecules, easily cause intractable problems including irreversible or competitive absorption on the stationary phase, decrease in column performance, and column contamination and clogging. Samples prior to injection should be as particle-free as possible, especially for a LVI method, as large amounts of sample matrices should be introduced into an analytical column after large volume injection [32]. Thus, removal of particles is an essential part of sample cleanup for HPLC analysis. Theoretically, the turbidity based on measuring the scattered light can indicate the concentration of small particles in the solutions. As expected, it was discovered that the precolumn Δp increased with a rise in the turbidity of supernatant as shown in Fig. 2. Furthermore, for a low level of turbidity (e.g., the turbidity value of 0.06 NTU), the precolumn could tolerate more than 50 large-volume injections without observable changes in pressure. The results suggested that turbidity could be used as a reliable and practical indicator for further investigation of cleanup efficiency, obtaining optimal UAMC conditions with good comparability with LVI-HPLC.

### 3.2.2. Influenced of ultrasound on cleanup efficiency

High-intensity ultrasonication, based on sound waves with frequencies ranging from 16 kHz to several hundred kHz, has been reported to improve surface hydrophobicity of proteins by exposing more hydrophobic groups to the solvent phase [41,42]. Therefore, ultrasound treatment might improve the transfer of proteins to the hydrophobic core of surfactant micelles, and consequently facilitate the removal of proteins from the aqueous supernatant. In addition, the mechanical effect of ultrasonic cavitation could accelerate the movement of TX-114 molecules and consequently facilitate micelle aggregation as well as larger micelle network formation, thereby giving rise to a strong viscosity enhancement of the surfactant-rich phase [40]. Due to a higher viscosity, the surfactant-rich phase becomes firmer and more stable, which is beneficial for phase separation and supernatant collection. As confirmed in Fig. 3 A, lower turbidities could be obtained with ultrasound treatment, suggesting a better cleanup performance. When the centrifuge tube was inverted to collect the supernatant, the surfactant-rich phase obtained with 2-min ultrasound treatment still adhered at the bottom due to its high viscosity, while a more flexible bottom phase without ultrasonication was observed under the same conditions (Fig. 3 A). The results indicated a synergistic effect between micelles and ultrasound on efficient matrix cleanup and phase separation. As shown in Fig. 3 B, turbidity could notably decrease within 4 min ultrasound treatment. Slightly longer ultrasound time was beneficial to increase the viscosity of surfactant-rich phase, which can facilitate phase separation and collection of supernatants. Thus, 8 min was finally selected.

### 3.2.3. Cleanup efficiency influenced by pretreated solution components

The concentration of formic acid plays a crucial role in matrix cleanup for two reasons. First, at a pH far from the isoelectric point (pI), the unfolding of proteins is induced due to the increased net charges and enhanced charge repulsion [43]. Therefore, surfactants are more likely to bind to the unfolded state of proteins [44], efficient removal of proteins could be achieved at lower pH. Second, a less polar aqueous medium owing to the addition of formic acid [45] might facilitate the migration of hydrophilic proteins such as albumin [39] to the surfactant-rich phase. As shown in Fig. 4 A1 and A2, the turbidity of the aqueous supernatant declined and finally reached equilibrium as the concentration of formic acid increased, and the turbidity obtained with two concentration levels of TX-114 and NaCl showed the same trend. Thus, concentrations higher than 3.6% would be suitable for obtaining good cleanup performance. At low concentrations, salts could neutralize charged side chains on amino acid residues and thus reduce electrostatic interactions [46], which might stabilize the native state of proteins against denaturation and hinder them from binding to surfactants. In contrast, a high concentration of salts could cause preferential hydration on the protein surface with the exposure of hydrophobic residues [46], and consequently promote the interaction between proteins and micelles. As shown in Fig. 4 B1 and B2, the turbidity rose and then declined within 5 min ultrasound treatment. Slightly longer ultrasound time was beneficial to increase the viscosity of the surfactant-rich phase, which can facilitate phase separation and collection of supernatants. Thus, 8 min was finally selected.

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**Fig. 2.** The relationship between turbidity and Δp, where Δp represents the variation of precolumn-pressure caused by the injection of supernatants (A). The corresponding phase separation phenomena of samples with different turbidities are shown in the original pictures (B).

**Fig. 3.** The turbidity of the diluted supernatant with or without ultrasound treatment (A) and with different ultrasonic time (B). The pictures in figure A show the corresponding surfactant-rich phase at the bottom of an inverted centrifuge tube under different conditions. The ultrasonic power was set at 200 W, and the temperature was 35°C.
separation. Therefore, NaCl concentrations ranging from 3.8 to 5.4% should be more preferable.

The content of surfactant was crucial to provide a sufficient quantity of micelles for the capture of sample matrices [23]. As shown in Fig. 4 C1 and C2, a TX-114 concentration of more than 5.4% might be adequate for sample cleanup.

3.3. Extraction efficiency

3.3.1. Single factor experiment

The contribution of the extraction process to method sensitivity and accuracy can be represented by extraction recovery ($E_{aq}$) and enrichment factor ($F_{enrichment}$), the ratio of the analyte concentration in extraction phase ($c_{aq}$) to that in samples ($c_{sample}$). They could be employed to investigate the influence of parameters on the extraction performance of UAMC. The phase ratio ($V_{surf}/V_{aq}$, the volume ratio of the surfactant-rich phase to the extraction phase) and the distribution coefficient between aqueous phase and surfactant-rich phase ($K_{aq/surf}$) are closely related to $E_{aq}$ and $F_{enrichment}$, and hence the experimental conditions that could cause a significant change in $V_{surf}/V_{aq}$ or $K_{aq/surf}$ should be the key parameters. According to Eqs (1)–(2), the $E_{aq}$ is positively related to $K_{aq/surf}$ and negatively related to $V_{surf}/V_{aq}$, while $F_{enrichment}$ is mainly related to $K_{aq/surf}$. Thus, the influence of parameters on $F_{enrichment}$ might show the similar trend to that on $K_{aq/surf}$, but their influence on $E_{aq}$ should be given the consideration of $K_{aq/surf}$ and $V_{surf}/V_{aq}$.

Micelles could have a dual effect on extraction performance. On the one hand, micelles dominate the distribution of analytes by capturing biomacromolecules such as proteins and deactivating analyte-binding sites of proteins [23]. Cefathiamidine, with a protein binding capacity of 23% [34], was released from the sample matrices to the aqueous supernatant with an adequate number of micelles, resulting in an increase in $K_{aq/surf}$. However, cefathiamidine might interact with the excess micelles, which reduces the extraction efficiency. As confirmed in Fig. 5 A1 and B1, $K_{aq/surf}$ and $F_{enrichment}$ increased when the TX-114 concentration ranged from 2.7% to 10.8%. A decline of $K_{aq/surf}$ and $F_{enrichment}$ was observed when the concentration exceeded 10.8%. On the other hand, increasing number of micelles could cause a rise in $V_{surf}/V_{aq}$, which decreases the $E_{aq}$. In the range of 2.7–10.8%, the influence of TX-114 concentration on $E_{aq}$ was a comprehensive result from $V_{surf}/V_{aq}$ and $K_{aq/surf}$, as the effects of these two factors offset each other according to Eq (1).

Salts with strong salting-out capacity play an important role in phase separation: the presence of salts could increase the incompatibility between the water structures in hydration shells of ions and micelles, which can reduce the concentration of “free water” in the surfactant-rich phase, and consequently reduce $V_{surf}/V_{aq}$ [47,48]. However, salts might have less effect on $K_{aq/surf}$. Thus, $E_{aq}$ was enhanced when the NaCl concentration ranged from 3.9 to 5.9% due to the notable decline in $V_{surf}/V_{aq}$ while $F_{enrichment}$ and $K_{aq/surf}$ changed slightly with increasing NaCl concentration (Fig. 5A2 and B2).

Since cefathiamidine is derived from 7-aminocephalosporanic acid (7-ACA) and sensitive to base, the influence of formic acid on analyte distribution is important. Increasing amount of formic acid might lead to
a less polar circumstance \[45\]. In addition, the more acidic environment facilitated the conversion of cefathiamidine ions to neutral species, and consequently enhanced the hydrophobicity of the analyte, and consequently reduced the retention of cefathiamidine in the aqueous supernatant. Therefore, \( K_{aq/surf} \) reduced with an increase in the concentration of formic acid (Fig. 5 B3), and consequently caused a decrease in extraction performance (Fig. 5 A3). The results in Fig. 5 A3 show that \( E_{aq} \) and \( F_{enrichment} \) notably declined when the concentration of formic acid increased, suggesting a slightly acidic environment would be preferable for extraction.

Furthermore, the influence of sample volume and ultrasound parameters was investigated. According to Eq (3) and Fig. S1, increasing the sample volume \( (V_{sample}) \) resulted in an increase in \( F_{enrichment} \) and consequently improved method sensitivity. However, excess amounts of sample matrices could lead to drop in \( E_{aq} \) and insufficient sample cleanup. In view of the compromise among sensitivity, cleanup efficiency and matrix effect, a sample volume of 300 μL was adopted. Ultrasound parameters including temperature, ultrasonic time and ultrasonic power did not significantly influence \( E_{aq} \) and \( F_{enrichment} \) (as shown in Fig. S2). These results might be attributed to the fast extraction equilibrium of UAMC. Although \( E_{aq} \) and \( F_{enrichment} \) could reach equilibrium within 2 min as shown in Fig. S2B, 8 min was recommended to obtain better phase separation and easier supernatant collection as discussed in section 3.2.2. Finally, 35 °C, 8 min and 200 W were selected to provide a mild but effective processing conditions for analyte extraction.

\[
E_{aq} = \frac{c_{aq}}{c_{aq}} \times \frac{V_{aq}}{V_{sample}} = \frac{1}{(V_{surf}/V_{aq})/K_{aq/surf} + 1} \quad (1)
\]

\[
K_{aq/surf} = \frac{c_{aq}}{c_{surf}} \quad (2)
\]

\[
F_{enrichment} = \frac{c_{aq}}{c_{sample}} = \left( \frac{1}{V_{surf}/K_{aq/surf} + V_{aq}} \right) \times V_{sample} \quad (3)
\]

3.3.2. Box-Behnken experiment

The concentrations of TX-114 (A, %), NaCl (B, %) and formic acid (C, %) were studied at three levels using a Box-Behnken design. As a key factor influencing method sensitivity and accuracy, the enrichment factor \( (F_{enrichment}) \) was used as the experimental response \( (Y) \) [23]. The BBD design matrix is shown in Table 3. A multiple regression model was performed to analyze the relationship between response and the
variables at the 95% confidence level (CL). The regression equation is given as Eq. 4 and the results of analysis of variable (ANOVA) are listed in Table 4 to assess the quality of the suggested model. The F-value of the model was 94.82 (p < 0.001), indicating that the suggested model was extremely significant. The ‘lack of fit F-value’ of 3.03 (p greater than 0.05) denotes that the lack of fit is not significantly related to pure error, implying satisfactory model fitness [49]. In addition, the coefficient of determination (R^2) was 0.9896, suggesting a good correlation between the model and the empirical data [50]. Furthermore, the “Adj-R^2” of 0.9791 was in good agreement with the “Pred-R^2” of 0.9812, indicating that the model without outfitting is reliable to explain the experiment [51]. In conclusion, the suggested model possessed good fitness and predictive ability.

A p-value<0.05 indicates that a model term is statistically significant at 95% CL. In this case, all model terms except A^2 were statistically significant; thus, A^2 was removed from the model to improve fitness. Two-dimensional contour plots and three-dimensional response surface plots were used to reveal the interactions between factors (Fig. 6) [52]. The absolute value of coefficients in regression equation evaluates the contribution of each term to F_enrichment [53]. According to Eq. 4 and Fig. 6, concentration of formic acid and its interactions with other factors had greater contributions. Therefore, the concentration of formic acid plays a more important role in the extraction efficiency, showing a negative correlation, which is in agreement with the conclusion of the single factor experiment.

Overall, the amount of formic acid is a critical factor both in analyte extraction and sample cleanup. To obtain both efficient extraction and good cleanup performance, 3.6% was selected as the optimized concentration of formic acid. According to Fig. 6B2 and B3, at the selected concentrations of formic acid, F_enrichment presents a downward trend with the increase in the concentration of TX-114 or NaCl. Finally, referring to the optimization results of cleanup efficiency, 5.4% of TX-114 and 3.8% of NaCl were selected. Under the optimized conditions, the average F_enrichment of ten validation tests was 0.167 (RSD < 1%). The relative error between the average F_enrichment value and the model prediction value was calculated to be 1.16%, indicating that the model is reliable and robust as it agrees well with the actuality.

\[ EF = 0.178 + 4.778 \times 10^{-3}A - 2.982 \times 10^{-5}B + 2.609 \times 10^{-3}C + 1.966 \times 10^{-5}AB - 5.432 \times 10^{-6}BC - 1.335 \times 10^{-5}AC + 4.083 \times 10^{-7}B^2 + 5.336 \times 10^{-10}C^2 \]

3.4. Large volume injection based on column-switching strategy

Direct large volume injection of sample matrix generally leads to degraded performance and shortened lifetime of the analytical column, and hence we have employed a column-switching strategy to solve this problem. As shown in Fig. 1B, due to the introduction of a weak mobile phase containing a low percentage of acetonitrile, the analytes could be focused on the precolumn, while most of the interfering residual surfactant micelles and other weakly retained matrix components were eluted to the waste. The elimination of interferences is based on the fast baseline separation of cefathiamidine from the sample matrix on the LVI-precolumn, which requires a proper composition of the mobile phase from pump 1. To monitor the outflow of cefathiamidine and matrices from the LVI-precolumn, the analytical column and the six-port value in Fig. 1B were removed, and the LVI-precolumn was directly connected to the autosampler and the PDA detector. Then, acetonitrile contents in the mobile phase from pump 1 were investigated and optimized. According to the chromatograms in Fig. 7A, lower acetonitrile contents in the mobile phase could increase the retention of cefathiamidine (peak 1), providing higher resolution between the analyte and impurities. Sufficient separation of cefathiamidine from matrices was observed when the acetonitrile content was decreased to 5%. According to the corresponding chromatogram (curve a in Fig. 7A), a suitable enrichment time should be 2.5 min, which could ensure the outflow of most matrix components and avoid analyte loss.

After 2.5 min of enrichment, analytes were eluted from the LVI-precolumn to the analytical column by switching the value from position 1 to 2, as shown in Fig. 1B. In Fig. 7B1, to monitor the elution process for seeking a proper elution time, the analytical column was removed, and the eluates from the LVI-precolumn were directly transferred into the PDA detector. According to the chromatograms in Fig. 7B1, after switching the value at 2.5 min, the transfer of most eluates could be completed within approximately 0.5 min. As further confirmed in Fig. 7B2, the intensity of the eluted cefathiamidine reached equilibrium after 0.5 min, and hence 1 min was selected for a more thorough elution. The valve was switched back to position 1 after 1-min elution, and then the subsequent LVI-precolumn regeneration and chromatographic separation procedures could be performed simultaneously to reduce time consumption.

The LVI method under the abovementioned conditions can tolerate at least 500-µL injection without peak broadening and impurity interference, indicating a large sensitivity enhancement over the conventional 10-µL injection method. The LVI-precolumn could be reused for more than 50 cycles without performance degradation due to high cleanup efficiency of UAMC.
3.5. Validation and application of UAMC-LVI-HPLC

3.5.1. Method validation

According to the ICH guidelines, the method was validated in four types of sample matrices: whole blood, plasma, serum and zebrafish. Typical chromatograms of blank and spiked samples are shown in Fig. 8. No significant response attributable to interfering components was observed in the retention time of cefathiamidine. The non-matrix-matched calibration curve ranged from 0.030 to 100 mg/L (0.15–489 μg/g for zebrafish samples) with good linearity (R² = 0.9999), which covered the possible concentration under a drug treatment. The LODs and LOQs of were calculated to be 0.0051 and 0.030 mg/L for whole blood, plasma and serum, respectively, and 0.038 and 0.15 μg/g for zebrafish. Recoveries of QC samples spiked at four levels were in the range of 97.6–109.7%, representing a satisfactory accuracy (Table 5). As listed in Table 5, the intraday and interday RSDs ranging from 0.5 to 4.9% indicated excellent precision, which was mainly credited to the simple, easy and repeatable operation of UAMC. In conclusion, the method enjoys a wide linear range, good linear correlation, satisfactory accuracy and excellent precision.

Compared with recently reported methods, UAMC-LVI-HPLC shows simplicity, superior linear range, competitive LOD, accuracy and precision. The LOQs of the proposed method are lower than those of HPLC methods [54,55] and comparable to those of most HPLC-MS/MS methods [56,57]. Moreover, all current methods concerning the analysis of cefathiamidine require matrix-matched calibration to reduce matrix interference, and sometimes internal standard calibration is also needed. These calibration methods easily cause problems such as matrix bias, increased workload or limited availability of suitable matrices and internal standards. In contrast, UAMC-LVI-HPLC has provided a non-matrix-matched calibration method without using an internal standard, where linear curves prepared in water were applicable to the analysis of all the above-mentioned sample types. This feature can avoid the abovementioned problems, simplify the procedures, shorten the analysis time and reduce the cost of labor and materials.

3.5.2. Application

The validated UAMC-LVI-HPLC method was applied in the analysis of cefathiamidine in zebrafish after drug treatment, as a challenging positive real sample. According to Commission 2002/657/EC [58], cefathiamidine was identified based on both the retention time and ultraviolet absorption spectra. As shown in Fig. S4, there was no obvious difference of the spectra between the real sample and the standard solution. Cefathiamidine in zebrafish treated at three different levels (0.28...
Fig. 7. The effects of time on matrix removal (A), analyte enrichment (A) and analyte elution (B1 and B2). After removing the analytical column and the six-port valve from the LVI-HPLC system, the spiked whole blood samples (Fig. 7A) flowed through a 10 μm C18 precolumn, and then directly a PDA detector for monitoring. The solutions are driven by pump 1 with the aqueous mobile phase containing 5%, 6%, 7%, 10% acetonitrile (curves a–d in Fig. 7A). After removing the analytical column from the LVI-HPLC system, the whole blood samples flowed through a 10 μm C18 precolumn for enrichment. Then, the valve was switched to position 2, and eluates from this LVI-precolumn were directly transferred to a PDA detector. The whole enrichment and elution process was monitored by the PDA detector, including the blank (curve a in Fig. 7B1) and spiked whole blood (curve b in Fig. 7B1). Fig. 7B2 shows the peak areas of cefathiamidine with different elution times when whole blood was spiked at 5 mg/L. Peak 1: cefathiamidine.

Fig. 8. Chromatograms for blank samples (a), spiked samples (b) and the standard solutions (c) analyzed by the UAMC-LVI-HPLC method (A–C are for whole blood, plasma, serum spiked at 30 mg/L, respectively, and D for zebrafish spiked at 150 μg/g) Peak 1: cefathiamidine. The volume ratio of acetonitrile to the aqueous solution of pump 2 was 16:84 for A or B and 15:85 for C or D.
Table 5  
Precision and accuracy for the determination of cefathiamidine in spiked whole blood, plasma, and zebrafish samples by the UAMC-LVI-HPLC method (intraday: n = 6; interday: n = 3 series per day, 3 days).

| Sample matrix | Spiked concentration | Interday RSD (%) | Interday RSD (%) | Mean recovery (%) |
|---------------|----------------------|------------------|------------------|-------------------|
| Whole blood   | LOQ                  | 4.9              | 2.6              | 103.3             |
|               | 0.09                 | 2.7              | 3.5              | 101.5             |
|               | 30                   | 3.0              | 1.4              | 98.2              |
|               | 80                   | 2.3              | 1.0              | 97.6              |
| Plasma        | LOQ                  | 2.1              | 1.3              | 108.6             |
|               | 0.09                 | 0.8              | 1.7              | 107.9             |
|               | 30                   | 2.5              | 1.0              | 107.9             |
|               | 80                   | 0.5              | 0.8              | 109.0             |
| Serum         | LOQ                  | 1.5              | 2.3              | 107.5             |
|               | 0.09                 | 1.3              | 1.0              | 109.7             |
|               | 30                   | 1.7              | 0.6              | 108.6             |
|               | 80                   | 1.5              | 0.7              | 108.5             |
| Zebrafish     | LOQ                  | 2.1              | 1.8              | 108.2             |
|               | 0.45                 | 4.5              | 4.0              | 107.0             |
|               | 150                  | 0.9              | 1.3              | 107.1             |
|               | 400                  | 1.0              | 0.7              | 107.2             |

* The spiked concentration unit for whole blood, plasma and serum samples is μg/L, and for zebrafish sample is μg/g.

mM, 1.0 mM and 2.0 mM) could be detected and calculated to be 0.683 μg/g, 2.182 μg/g and 9.757 μg/g with RSDs (n = 3) of 1.3%, 2.6% and 6.4%, respectively. No significant interference was detected in the retention time of cefathiamidine in the chromatogram of the control group (Fig. S5). The results indicate the excellent practical applicability of UAMC-LVI-HPLC method.

4. Conclusion

Herein a novel UAMC-LVI-HPLC method was developed and successfully applied to the analysis of cefathiamidine in complex biological samples such as whole blood, plasma, serum and zebrafish. UAMC enabled synchronous efficient sample cleanup and analyte extraction by means of fast phase separation within 8 min. To quantitively evaluate the cleanup efficiency, we used turbidity as an indicator, which can successfully denote the compatibility between sample cleanup conditions and chromatographic analysis. The turbidity results showed that ultrasound could facilitate matrix removal by promoting phase separation and micelle-matrix interaction. The extraction performance results indicated a short extraction equilibrium time of approximately 2 min. The fast simple operation was beneficial to high-throughput sample preparation. UAMC was proven to be applicable to the analysis of polar analytes in both liquid and solid biological samples. In addition, the impressive cleanup capability of UAMC allows good compatibility with LVI-HPLC, providing a 50-fold enhancement in the sensitivity of the method. The good performance of UAMC-LVI-HPLC in method validation and real sample application indicates that the method has wide linear ranges, good linear correlation, satisfactory accuracy and excellent precision. Moreover, from a practical point of view, UAMC-LVI-HPLC is simple, cost-effective and easy to operate in conventional labs. Therefore, it is expected to be a promising tool for TDM and pharmacokinetic and toxicology studies in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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