Journal of Chromatographic Science, 2020, Vol. 58, No. 6, 576–584
doi: 10.1093/chromsci/bmaa021
Advance Access Publication Date: 25 May 2020
Article

Article

Collection and Separation of Fleroxacin and Ciprofloxacin in Ultrasound-Assisted Ionic Liquid Salting-Out Microextraction System

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Received 22 July 2019; Editorial Decision 17 April 2020

Abstract

An ultrasound-assisted ionic liquid (IL) salting-out microextraction system was developed and applied for the extraction of quinolone antibiotics from urine. A precipitate was formed from the salt and IL, and it acted as the sorbent for the analytes. The precipitate containing the analyte was separated by filtration, redissolved, and the solution then was evaporated. The resulting extract was redissolved for high-performance liquid chromatographic analysis. Several parameters, including type and volume of IL, the type and amount of salts, sample pH, temperature and extraction time were optimized. Under the optimal experimental conditions, the limits of detection for fleroxacin and ciprofloxacin were 3.12 and 4.97 μg L^{-1}, respectively. When the present method was applied to real urine sample analysis, the analyte recoveries ranged from 82.3 to 106.8%. This ultrasound-assisted IL salting-out microextraction system had the characteristics of high recoveries, shorter separation time and easy-to-perform collection procedure, which yielded the method to have potential for wide application.

Introduction

Antibiotics have been widely used in recent decades for treating a number of human and animal ailments (1). In addition, antibiotics have played an integral role in prophylactic treatments. Quinolones (QNs) are an important class of synthetic antibiotics that have broad-spectrum activity against both gram-positive and gram-negative bacteria. QNs are also widely used in animal farming and food production industries specializing in animal products. However, QNs are not efficiently absorbed in the animal gut and, in fact, up to 30–90% of the parent compound being excreted via feces or urine (2). To protect consumer health, the European Union has established maximum residue limits (MRL) for antibiotics, including QNs, as administered to animals in the farming industry (European Commission, 2010) (3). QNs belong to the pyruvate antibiotic derivative family in chemical structure. According to synthetic times and different antibacterial activities, antibiotic development has gone through five generations. Drugs represented by nalidixic acid and oxalic acid were the first-generation antibiotics synthesized in the 1960s (4). Drugs represented by pipemidic acid and flumequine were second-generation antibiotics, synthesized in the early 1970s. Drugs represented by norfloxacin were the third-generation antibiotics, synthesized in the late 1970s. A common feature of the third-generation QNs was the introduction of fluorine atoms at the naphthyridine ring 6-position and the introduction of a piperazinyl ring group or pyrrolyl group at the 6-position. Drugs represented by sparfloxacin and gatifloxacin were the fourth-generation antibiotics in the 1990s. LM-K antibiotics synthesized by Japanese scientists in 2002 were the fifth-generation antibiotics and are still in the clinical research stage. At this stage, the third- and fourth-generation QN antibiotics, including norfloxacin and sparfloxacin, are the most used in clinical applications (5).

To ensure accurate detection of trace substances, analytes need to be enriched before analysis. Different procedures have been discussed in the literature regarding processes for QN determination (6, 7). The most popular method for determination of QNs in different matrices has been high-performance liquid chromatography (HPLC) with different detection modes, such as fluorescence (8), DAD (9, 10) ultraviolet (UV) (11) and mass spectrometry (MS) (12, 13). To effectively detect low antibiotic content in samples, proper sample preparation is required in terms of clean-up and extraction processes. Liquid–liquid extraction (LLE) (14) and solid-phase extraction (SPE) (15) are the most commonly employed. At present, some techniques were developed for preparing samples for QN extraction, such as solid-phase microextraction (SPME) (16), single drop microextraction (17), molecularly imprinted solid-phase extraction (MISPE) (18), dispersive liquid–liquid microextraction (DLLME) (19), aqueous two-phase extraction (ATPs) (20) and ionic liquid (IL) dispersive liquid–liquid microextraction (IL-DLLME) (21).

In recent years, IL/salt aqueous phase systems have been extensively studied (22). Another system of IL salting out has rarely been reported, which takes advantage of ILs and salts being able to form homogeneous precipitates, which have adsorption properties and can be used for enrichment and separation of samples. The principle of salting-out microextraction is based on the hydration of salt ions, as water hydration layers, originally "hydrated" with organic solvents and thus releasing organic solvent molecules in a two-phase salt-dissociating system for organic solvents and forming inorganic salts. When a salt is dissolved in an aqueous solution, its ions are surrounded by water hydration layers, a process known as ionic hydration. In the present case, when an inorganic salt is added to an aqueous solution of an IL, the two solutes compete for the solvent molecules. The competition is won by the inorganic ions, by having a stronger interaction with the solvent, and those of the IL lose. There is a "migration" of solvent molecules away from the ions of the IL toward those of the inorganic salt, which, in turn, decreases the hydration and therefore the solubility of the IL in water (23). Utilizing the hydration of salt ions, the salt ions capture the water molecules hydrated by the organic solvent to release the organic solvent molecules, forming a two-phase salting-out system of organic solvent and inorganic salt. The analytes in the sample enter into the two-phase system due to surface properties, charge action and the presence of various forces, such as hydrogen and ionic bonds, and environmental factors, such as concentration, temperature and pH. The analytes were easily distributed between the two phases.

In this study, an ultrasound-assisted IL salting-out microextraction system was examined for the extraction and preconcentration of antibiotics from urine. In the IL/IL dispersive liquid–liquid microextraction step, the homogeneous precipitate formed from ILs and salts was used for the extraction and separation of analytes from samples. To the best of our knowledge, for the first time, an ultrasound-assisted IL salting-out microextraction system was developed and applied to the analysis of QNs in urine, demonstrating that the method could be applied for analyte determination in similar samples.

Experimental

Reagents and chemicals

Ciprofloxacin and fleroxacin were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). QN structures and properties are shown in Figure 1 and purities both > 98%. Stock solutions were prepared at 500 μg mL^{-1} using acetonitrile (chromatographic grade; Fisher Scientific U.K., Ltd., Loughborough, UK) containing 5.24 mM acetic acid. For experiments, these chemicals were first diluted using pure distilled water from a Milli-Q water purification system (Millipore Corp., Billerica, MA, USA). Other chemicals such as 1-butyl-3-methylimidazolium tetrafluoroborate ($[C_4MIM][BF_4]$, purity > 99%), analytical-reagent grade formic acid (purity > 99%) and potassium hydrogen phosphate anhydrous (purity > 98%) were purchased from Shanghai Cheng Jie Chemical Co., Ltd. (Shanghai, China) and Aladdin Chemistry Co., Ltd. (Shanghai, China), respectively. Tripotassium orthophosphate and potassium phosphate monobasic with purities > 99% were purchased from Pengcai Chemistry Co., Ltd. (Langfang, China).

Instruments

A 1100 series liquid chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with UV detector and quaternary gradient pump was used, along with a Zorbax Eclipse Plus-C_{18} column (150 × 4.6 mm, 3.5 μm; Agilent Technologies, Inc.) and a C_{18} guard column (7.5 cm × 2.1 mm I.D., 5 μm particle size). The HPLC-UV system was utilized for determining refinements in other preparations and experimental conditions. The KQ-100DE ultrasonic cleaner was bought from Kunshan Ultrasonic Instrument Co., Ltd. (Kunshan, China) with the frequency and output power at 40 kHz and 100 W, respectively. The SH-39 oscillator was bought from Shanghai Zhenghui Instrument Co., Ltd. (Shanghai, China). An RE-52AA vacuum rotary evaporator (Xi'an Heb Biotechnology Co., Ltd., Shaanxi, China) was also used.

Samples

In this study, five kinds of samples were used, including human urine samples (Sample 1) obtained from healthy volunteers not taking any drugs. Rabbit urine samples (Sample 2) were obtained from a local animal experiment center. Fresh human urine samples (Sample 3) were obtained from a local hospital, river water (Sample 4) from the local Yitong River and running water (Sample 5) from Jilin University. Urine samples were obtained after fasting for 12 h. Except for the experiments mentioned in Section 3.3, which were performed with all five samples, all other results were obtained with Sample 1.

Urine pretreatment

An 8.0 mL volume of urine sample was added to a 15 mL polypropylene tube. Spiked samples were prepared by spiking mixed working solution into urine samples. Then, a 1.0 mL volume of acetonitrile was added and the mixture shaken and centrifuged at 15,000 rpm for 10 min. The resulting supernatant was then filtered by passage through 0.45 μm filters, which helped to eliminate denatured proteins from the mixture (24), and then stored at 4°C.

Running water

Spiked river water samples containing QNs were prepared by spiking working solutions into water samples. The resulting solution was referred to as the sample solution, filtered through 0.45 m filters, and then stored at 4°C.

HPLC-UV conditions

The mobile phase consisted of acetonitrile (A) and aqueous solution (3 mM $[C_2MIM][BF_4]$, 10 mM ammonium acetate and glacial acetic acid adjusted to pH 2.7) (B). The gradient flow program was as 0–10 min, 11–15% A; 10–15 min, 15% A; 15–18 min, 15–20%

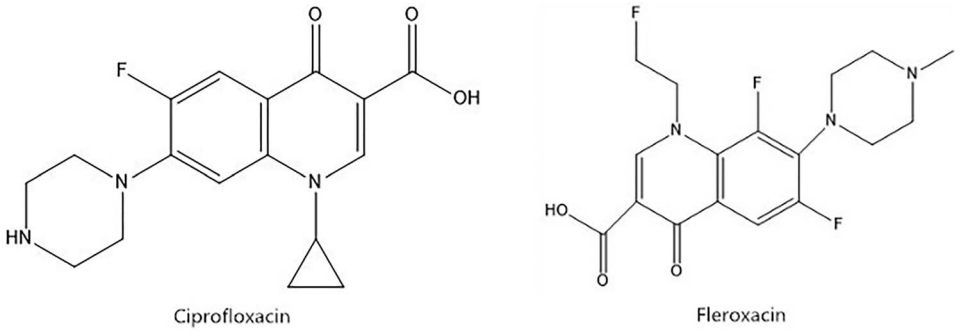

Figure 1. Chemical structures of QNs.

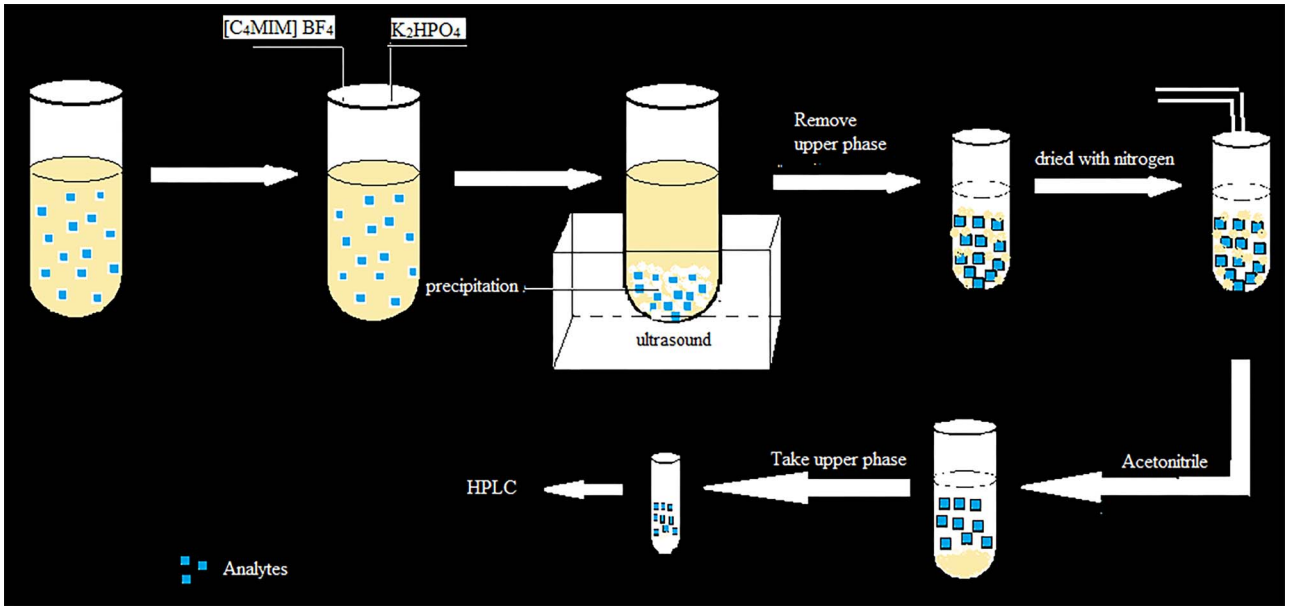

Figure 2. Extraction procedure.

A; 18–25 min, 20–11%. The mobile phase flow rate was held at 0.5 mL min^{-1} and column temperature at 30°C. The injection volume of analytical solution was 20 µL, monitoring wavelength at 277 nm, and reference wavelength and spectral bandwidth at 360 and 4 nm, respectively.

Extraction procedure

The sample solutions produced above and 260 µL of $[C_4MIM]BF_4$ were placed in a 15 mL centrifuge tube and 2.30 g of K_2HPO_4 added under ultrasound conditions to produce a homogeneous precipitation. After ultrasonic treatment for 4 min and centrifugation at 6000 rpm for 5 min, most of the solution was recovered, the precipitation dried under nitrogen, and then dissolved with 100 µL acetonitrile. The resulting solution was filtered by 0.22 µm PTFE filter membrane before analysis; the extraction process is shown in Figure 2.

Results

The optimized conditions for the salting out of the IL liquid–liquid ultrasonic extraction of fleroxacin and ciprofloxacin in real urine sample extracted these analytes at > 82.3% yield. The optimal conditions for the extraction of 260 µL of $[C_4MIM][BF_4]$ included 2.3 g of K_2HPO_4, 4 min of ultrasonic treatment and 20°C temperature.

Discussion

Types of ILs and salts

Characteristics of ILs, such as solubility in water, viscosity, extraction capacity and chromatographic behavior, played a key role in influencing the enrichment factor and recovery. It was necessary to consider the relationship of the extraction capacity and IL types. A series short chain and water-soluble ILs such as $[C_4MIM][BF_4]$, $[C_6MIM][BF_4]$ and $[C_6MIM]Cl$ were used to study salting-out IL extraction. All of them and KH_2PO_4 can produce salt precipitation, however, the introduction of salts, such as K_3PO_4 and NaCl, cannot. The precipitation system consisting of $[C_4MIM][BF_4]$ and KH_2PO_4 was the most stable. Therefore, $[C_4MIM][BF_4]$ was selected here.

Volume of $[C_4MIM][BF_4]$

The volume of $[C_4MIM][BF_4]$ directly affected the formation of the salting-out homogenous extraction and analyte extraction. With increased $[C_4MIM][BF_4]$ volume, precipitation in the solution was

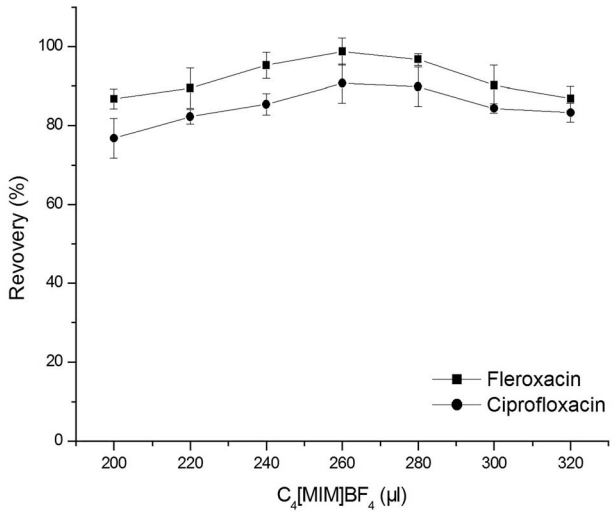

Figure 3. Effects of $C_4[MIM][BF_4]$ volume on analyte recoveries; sample amount: 8 mL, K_2HPO_4 amount: 2.3 g, ultrasonic time: 3 min and temperature: 20°C.

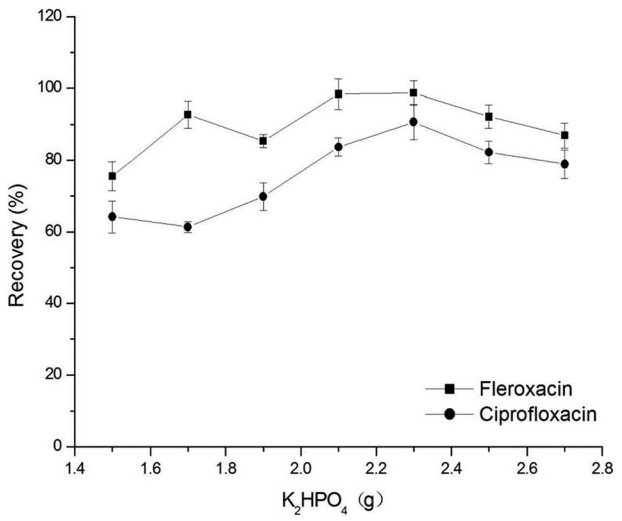

Figure 4. Effects of salt amounts on analyte recoveries; sample amount: 8 mL, $C_4[MIM][BF_4]$: 260 μL, ultrasonic time: 3 min and temperature: 20°C.

observed and the analytes enriched. The effects of $[C_4MIM][BF_4]$ volume on QN determination was evaluated by performing a series of experiments by adding different IL amounts (200–320 μL). As shown in Figure 3, the recoveries of analytes sharply increased and then slightly decreased with further increased $[C_4MIM][BF_4]$, being the highest when the volume was 260 μL (Figure 3). Therefore, 260 μL was selected as the optimal $[C_4MIM][BF_4]$ volume.

Amount of K_2HPO_4

A salting-out method is a highly popular mechanism used for improving efficiency in extraction processes, particularly in microextractions and for polar compounds. In the present process, the K_2HPO_4 quantity affected the charges of the salt and IL anions. This, in turn, resulted in creating a homogeneous salting-out system. Thus, here, K_2HPO_4 was used in quantities ranging from 1.5 to 2.7 g, to evaluate the influence of this salt's addition on the overall extraction process and its concurrent efficiency (Figure 4). It was evident that this salt addition improved extraction efficiency, reaching a maximum at 2.3 g of K_2HPO_4, above which its benefit decreased. This decrease was possibly because excess homogeneous precipitation tended to embed the analytes and suppress electrostatic interactions between salt ions and analytes in solution. On the basis of these results, 2.3 g K_2HPO_4 was chosen as optimal for succeeding experiments.

Effects of ultrasound extraction time

On a theoretical basis, increasing the time for ultrasound extraction would produce favorable results by achieving partition equilibrium for target analytes and could thus enhance analyte recovery as well. Therefore, in experiments, the effects of increased ultrasonication time on attained yields were studied over a range of 1–20 min (Figure 5). Due to ultrasound itself heating the system, ice water was added to keep the extraction temperature constant. The results showed that the highest yield was at 4 min, after which the yield decreased. This was probably because the precipitate structure was relatively loose and disassembled to some degree with excessive ultrasonication (25, 26). This implied that increased precipitate size increased the mass transfer rate due to increased contact area with

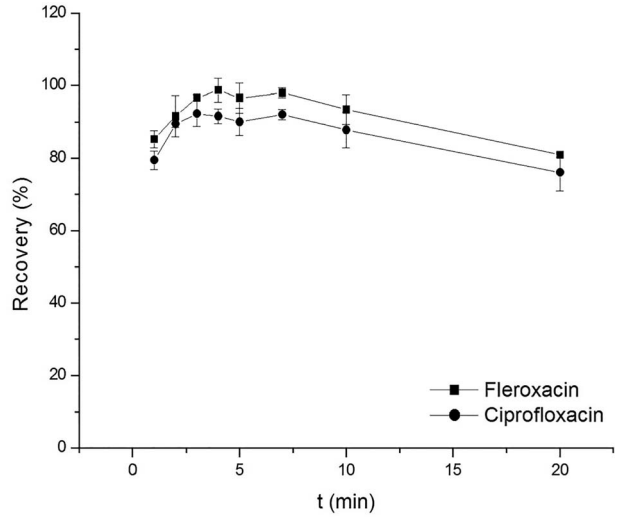

Figure 5. Effects of extraction time on analyte recoveries; sample amount: 8 mL, $C_4[MIM]BF_4$: 260 μL, K_2HPO_4 amount: 2.3 g and temperature: 20°C.

surrounding solution molecules, which led to an equilibrium state in as little as 4 min. With ultrasonic times > 4 min, the extraction system ultrasound vibration increased, resulting in increased precipitate dissolution, thus decreasing target analyte recoveries. As a result, 4 min of ultrasound time was selected as the optimal time for the ultrasound extraction step.

Extraction temperature

Temperature plays an important role in influencing solubility and mass transfer. Hence, the influence of temperature on recoveries from the extraction process was examined from 10 to 50°C. The temperature was held constant by adding hot water or ice water as the thermometer indicated. Extraction recoveries were found to increase from 10 to 20°C and then decrease from 20 to 50°C (Figure 6). This decrease was probably because precipitate solubility increased with increased temperature, resulting in sample analyte losses.

Table I. Regression equations, LOD and LOQ for HPLC

Compound	Correlation coefficient (r)	Regression equation ($n = 5$)	Linear range (μg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})	Intra day precision (RSD, %, $n = 5$)	Inter day precision (RSD, %, $n = 5$)
Fleroxacin	0.9991	$A = (-4.53 \pm 7.07^a) + (1.13 \pm 0.027^b)c$	12.4–381.6	3.12	10.40	2.8	3.9
Ciprofloxacin	0.9997	$A = (-2.70 \pm 1.39^a) + (0.30 \pm 0.0053^b)c$	17.7.–396.0	4.97	16.57	4.2	3.6

[a,b]Standard deviation of slope and intercept.

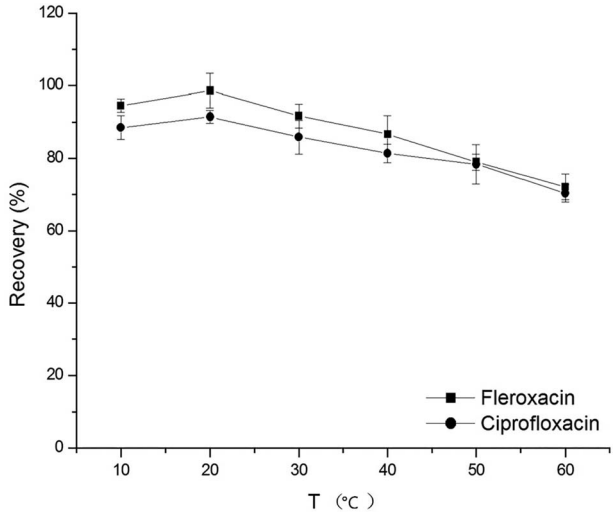

Figure 6. Effects of temperature on analyte recoveries; sample amount: 8 mL, C_4[MIM][BF_4]: 260 μL, K_2HPO_4 amount: 2.3 g and ultrasonic time: 3 min.

Method validation

The method was validated by examining several parameters, including specificity, limit of detection (LOD), limit of quantitation (LOQ), linear range, precision and accuracy. QNs were determined in samples under the above optimized conditions. HPLC chromatograms of blank and spiked urine samples were compared to evaluate specificity (Figure 7).

LOD, LOQ and quantification

Working standard curves represented the analyte quantities found in the samples. The linearity in these findings was evaluated using the present optimal conditions and the results used to generate linear regression equations and correlation coefficients (Table I). Notably, the correlation coefficients were (r) ≥ 0.9991 and favorable linearities obtained. LOD values, with a signal-to-noise ratio of 3 (Table I), fell within the range of 3.12–4.97 μg L^{-1}. Such low values indicated high applicability of the process for precise detection and evaluation of QN antibiotics. LOQs, with a signal-to-noise ratio of 10 (Table I), were found to fall within the range of 10.40–16.57 μg L^{-1}. As these values were found to be lower than MRLs, the present method was deemed feasible, valid and practical for other applications.

Precision

The precision and recovery potential of the present method was tested by calculating relative standard deviations (RSDs) for both intraday and interday tests (Table II). For evaluating intraday precision, five replicates of samples were examined within a day. For interday precision, samples were studied once a day for five consecutive days. It was clear that this method possessed excellent applicability in both time frames. The yield for recoveries of all antibiotics ranged from 82.3 to 106.8% for two concentrations.

Robustness

A Plackett–Burman design (3 factors and 2 levels, $N = 4$) for the evaluation of robustness effects was applied. The three factors were the [C_4MIM][BF_4] volume, K_2HPO_4 amount and ultrasound extraction time. The experiments were carried out in two replicates and the obtained analyte recoveries listed in Table III. The t-test was used to evaluate robustness. The equation for calculating "t" is listed below (31).

$$t = \frac{\text{average effect}}{2\sqrt{\left[\sum d^2/(N-1)\right](N/8)}/\sqrt{2N}}$$

The t-test results are shown in Table III and average recoveries calculated based on the results given in Table IV. The effects of each factor were the difference between recoveries. The term "d" represents the difference between effects and "N" the number of experiments. All t-values were < 2.67, with the 5% critical t-value associated with 3 degrees of freedom. Based on this statistical analysis, the robustness of the method appeared acceptable.

Analysis of real samples

The utility of the method developed here was then determined by applying it in QN residue determinations of samples of human, rabbit and hospital urine and two river water samples (samples 1–5, respectively; Table V). Clearly, this method produced good yields (85.3–105.2%) and fair precision (≤6.8%). Hence, this method produced fair accuracy for the two examined concentrations.

Comparison of the present method with other extraction methods

The present method was further examined by analyzing, for comparison, three kinds of samples by salting out LLE (14), SPE (28, 32) and DLLME (27). From these results, the yields obtained by the present method were slightly higher than the other methods and the LOD of the present method satisfactory, particularly as UV detectors are less expensive, and the method thus suitable for promotion. Compared with SPE and LLE, which use large amounts of organic solvent (4–400 mL) for extraction, the present method used a green solvent

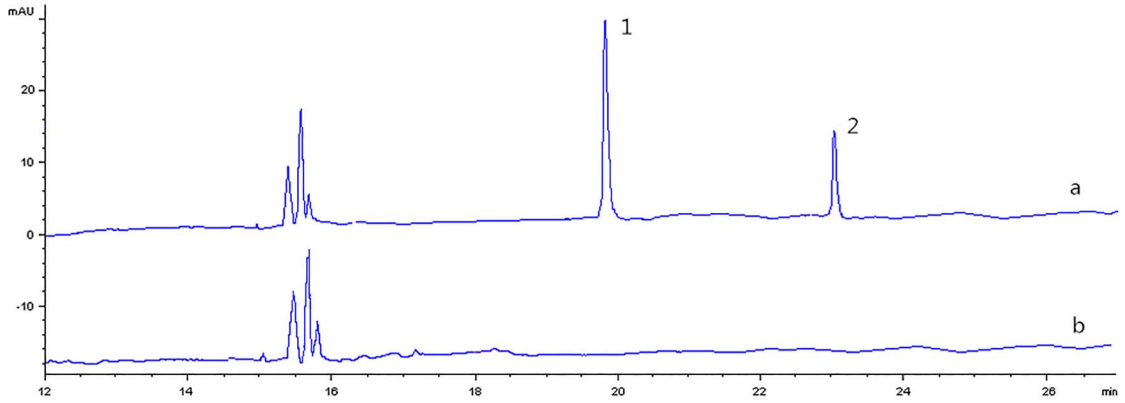

Figure 7. Chromatograms of spiked urine (a) and urine samples (b); ciprofloxacin (1) and fleroxacin (2).

Table II. The recoveries of the analytes in spiked Sample 1

Add (μg L^{-1})	Stored time (weeks)	Fleroxacin		Ciprofloxacin	
		Recovery (%)	RSD (% $n = 5$)	Recovery (%)	RSD (% $n = 5$)
50	1	96.4	3.0	92.4	1.7
	2	96.2	5.8	93.7	6.5
	3	91.3	2.9	88.5	3.1
	4	102.2	4.1	90.3	5.7
	6	93.1	6.7	91.9	4.0
	8	95.7	4.5	87.9	5.9
100	1	98.4	5.4	91.0	4.1
	2	94.1	7.1	95.8	6.2
	3	100.8	6.5	92.9	7.4
	4	95.9	2.4	87.9	2.5
	6	100.0	8.6	93.7	5.1
	8	99.7	5.6	88.1	3.8
150	1	106.8	6.8	93.6	6.4
	2	95.0	7.7	87.1	8.2
	3	97.9	1.5	89.4	3.5
	4	99.8	3.2	93.5	2.1
	6	98.5	4.8	95.2	6.0
	8	101.1	2.6	82.3	5.3

Table III. Arithmetic treatment of recovery data

Factor	Level	Fleroxacin							Ciprofloxacin						
		First data set		Second data set		A E	d	t	First data set		Second data set		A E	d	t
		AR (%)	Effect	AR (%)	Effect				AR (%)	Effect	AR (%)	Effect			
C_4[MIM]BF_4	260	99.1	2.4	97.8	−0.2	1.1	2.6	1.03	92.6	2.8	91.5	1.2	2	1.6	2.67
(μL)	280	96.7		98					89.8		90.3				
K_2HPO_4 (g)	2.3	98.7	1.6	96.5	−0.9	0.35	2.5	0.33	89.9	2.6	91	1.2	1.9	1.4	2.54
	2.1	97.1		97.4					87.3		89.8				
t (min)	4	97.9	1.3	98.1	3	2.15	−0.85	2.01	91.5	1.4	92.5	−0.1	0.65	1.5	0.87
	3	96.6		95.1					90.1		92.6				

d: difference between effects, *t*: *t*-values of *t*-test, AE: average effect, AR: average recovery.

Table IV. Comparison of the present method with other extraction methods

		Salting-out IL-DLLME	Salting-out LLE (14)	DLLME (27)	SPE (28)	SPE-Cigarette filters (29)	MISPE (18)	SPE (32)
Extraction yield (%)	Fleroxacin	102.3	–	104	94	94.1	–	–
	Ciprofloxacin	96.7	83.97	89	90	91.2	86.8	84
Extraction time (min)		3	10	3	30	83	60	6
Sample		Urine	Milk	Water	Milk	Waste water	Urine	urine
Sample amount (mL)		8	6	5	2	500	4	5
Extraction solvent		$[C_4MIM]BF_4$	Al^{3+} solution and ammonium sulfate	CHCl3	Acetonitrile	Methanol	Acetonitrile	Methanol and tetrahydrofuran
Solvent volume (mL)		0.26	0.1	0.685	6.0	4	400	5
Detector		UV	FL	DAD	MS	UV	DAD	UV
LODs (μg L^{-1})	Fleroxacin	3.12	–	7.81	0.11	2–5	–	–
	Ciprofloxacin	4.97	10	26.3	0.16	2–5	64	30

Table V. Analytical results of samples ($n = 5$)

Sample	Added (μg L^{-1})	Fleroxacin		Ciprofloxacin	
		Recovery (%)	RSD (% $n = 5$)	Recovery (%)	RSD (% $n = 5$)
Sample 1	100	99.6	4.5	89.2	3.6
	150	102.3	3.9	96.7	2.8
Sample 2	100	98.5	3.6	85.3	4.9
	150	104.8	6.8	95.7	5.2
Sample 3	100	97.7	2.0	97.9	6.1
	150	99.0	4.4	87.1	2.7
Sample 4	100	98.1	0.8	88.8	5.0
	150	103.0	5.3	100.1	1.7
Sample 5	100	105.2	2.1	97.8	1.6
	150	95.3	2.5	90.2	3.8

$[C_4MIM][BF_4]$ in smaller volume, shorter extraction time, and was overall environment friendly. Considering these advantages, this IL-based ultrasound-assisted extraction was a satisfactory method.

Conclusion

In this study, an ultrasound-assisted IL salting-out microextraction system was successfully applied to the extraction of QN antibiotics from urine samples. This system was then utilized to extract, separate and enrich QNs from urine in a single-step process. IL was used as a component of the mobile phase, which reduced peak tailing, thus improving target analyte separation (30). The method simplified sample treatment to a great degree. Furthermore, it had the advantages of reduced pretreatment time, enhanced extraction efficiency, significantly reduced RSD and ensured successful sample treatment in only a few minutes. It provided a simple, sensitive, economical and green method, and was successfully applied to urine for analysis of QNs. Hence, this method was recommended here for use in future applications for extraction, separation and concentration experiments of QNs antibiotics with differing experimental conditions in other similar samples.

Compliance with ethical standards

Conflict of Interest

None of the authors has any conflict of interest to disclose.

Funding

This work was supported by Jilin Province Science and Technology Development Project (No. 20190201244JC).

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

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