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Short communication

Application of single drop liquid–liquid–liquid microextraction for the determination of fluoroquinolones in human urine by capillary electrophoresis

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ABSTRACT

A simple and novel method of single drop liquid–liquid–liquid microextraction (SD-LLLME) coupled with capillary electrophoresis (CE) for the determination of six fluoroquinolones (FQs) was developed. The method was eventually applied to extraction and preconcentration of FQs in human urine samples. Good linear relationships were obtained for all analytes in a range of 40–1000 $\mu g\,L^{-1}$ with the correlation coefficients from 0.9913 to 0.9995. The limit of detections (LODs) varied from 7.4 to 31.5 $\mu g\,L^{-1}$ at a signal-to-noise (S/N) of 3. The recoveries at two spiking levels were 81.8–104.9% with relative standard deviations <8.3%.

1. Introduction

Fluoroquinolones (FQs) are a group of relatively new, highly potent, synthetic chemotherapeutic agents owing to a broad spectrum of activity against both gram-positive and gram-negative bacteria [1]. Nowadays, they have been the most extensively used drugs in the therapy of bacterial infections in humans as well as veterinary medicine [2]. Their antibacterial activity is based on a selective inhibition of bacterial DNA synthesis and producing bacterial death [3]. However, the misuse of these medicines may be responsible for increased concerns on public health, such as allergic reactions and antibiotic resistance [4]. Therefore, sensitive and selective methods for the determination of FQs in biological fluids are highly advisable.

Early publications have described techniques for FQs analysis in biological fluids including liquid chromatography (LC) [5,6], high-performance immunoaffinity chromatography (HPIAC) [7] and CE [8–12]. Among various types of methods, CE has been widely accepted due to the low sample consumption, high separation efficiency and fast analysis speed. However, the main drawback of CE is the poor concentration sensitivity because of the trace injection volume and the short light optical path in the most commonly used ultraviolet–visible (UV–vis) detection. To overcome this deficiency, on-line preconcentration techniques [13,14] such as stacking and sweeping or other more sensitive detectors such as mass spectrometry (MS) [10] and laser-induced fluorescence [11,12] are adopted before CE analysis. However, the sensitive instruments are not widespread due to more expensive compared with the cost of CE-UV for a common laboratory. No matter which way is adopted, sample pretreatment steps are also unavoidable to clean up sample matrices before analysis.

As most common sample pretreatment techniques, liquid–liquid extraction (LLE) and solid phase extraction (SPE) have been used for clean-up and preconcentration of FQs [9,15]. However, LLE is time-consuming, tedious and requires large volumes of high-purity solvents [16]. Although SPE is less time-consuming than LLE, it still requires toxic organic solvents for the elution step [17,18]. Solid-phase microextraction (SPME) has been applied for the determination of FQs because it is solvent-free, relatively fast, portable and easy to use [19,20]. Nevertheless, SPME suffers from some drawbacks: its fiber is fragile and has limited lifetime and sample carry-over is also a problem [21].

In order to overcome these problems, liquid-phase microextraction (LPME) has emerged. It is a simple, inexpensive sample pretreatment procedure and compatible with gas chromatography (GC) and HPLC. Recently, three-phase LPME, which consists of hollow fiber liquid–liquid–liquid microextraction (HF-LLLME) and SD-LLLME, is developed with a view to expanding the scope of the applications in CE and HPLC analysis. And the HF-LLLME has been successfully utilized for the clean up and pre-concentration of FQs from water [22]. As only small sample volumes are injected, SD-

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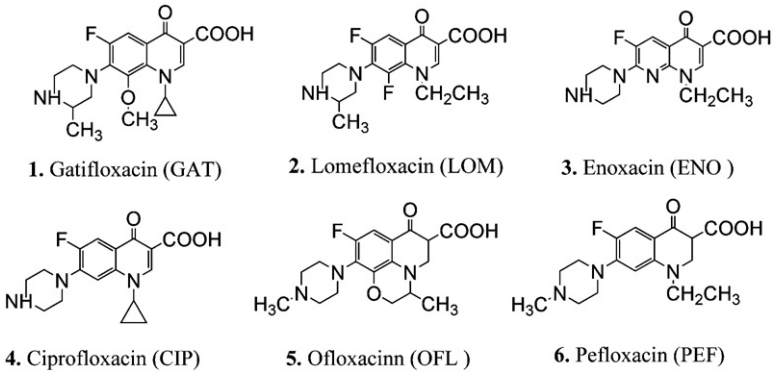

Fig. 1. Chemical structures of the fluoroquinolones.

LLLME is particularly well suited for CE. Nevertheless, no reports based on SD-LLLME coupled with CE focusing analysis of FQs were found.

In this work, SD-LLLME was applied for clean-up and preconcentration of six FQs prior to their determination by CE. The procedure adopted a mixture of solvent heavier than water floating on the top of aqueous sample as solvent layer and successfully applied to its determination in urine samples for the first time.

2. Experimental

2.1. Reagents and materials

Gatifloxacin (GAT), lomefloxacin (LOM), enoxacin (ENO), ciprofloxacin (CIP), ofloxacin (OFL) and pefloxacin (PEF) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). For their chemical structures, see Fig. 1. The 1 mg mL^{-1} individual stock solutions of the analytes were prepared in 0.01 M sodium hydroxide. Working solutions were filtered with 0.45 μm polyether sulfone filters (Xingya Purifying Materials Factory, Shanghai, China).

Disodium tetraborate, boric acid, disodium hydrogen phosphate, sodium chloride, sodium dihydrogen phosphate, dichloromethane (DCM) and toluene were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Sodium hydroxide, *n*-octanol, ethyl acetate and xylene were purchased from Guangzhou Chemical Reagent Plant (Guangzhou, China). All reagents were of analytical grade. Deionized water (18 Ω cm) was prepared by a Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Instruments

A CL1030 capillary electrophoresis system (Cailu, Beijing, China) equipped with a UV detector and hydrodynamic injection system was used for the determinations. A fused silica separation capillary of 50 cm (41 cm effective length) × 50 μm I.D. × 375 μm O.D. (Yongnian, Hebei, China) with 0.3 cm of detection window was used throughout the study. The data acquisition was carried out with a HW-2000 Chromatography Workstation (Qianpu, Shanghai, China).

A PHS-3CA precision pH meter (Dapu, Shanghai, China) was used throughout the experiment.

2.3. Extraction procedure

A 4.0 mL vial with a stir bar was placed on a magnetic stirring/hot plate (PC-420D, Corning Inc., USA). Then, 3.5 mL of aqueous sample solution containing GAT, LOM, ENO, CIP, OFL and PEF was transferred into the vial as donor phase (0.02 M NaH_2PO_4, pH 7.0). The donor solution was prepared by dissolving an appropriate amount of NaH_2PO_4 in water and adjusting to expected pH value by adding dropwise 0.1 M sodium hydroxide. A 350 μL organic phase (DCM:toluene, 1:1, v/v) was carefully added on the top of the donor phase to form the solvent layer. Then, 1 μL of 0.1 M NaOH was immersed into the organic phase as acceptor phase. During extraction, the solution was stirred at 450 rpm. After 40 min extraction, the acceptor phase was retracted into the microsyringe and transferred into a microvial. Then, the sample was introduced into capillary electrophoresis for analysis (Supplementary Fig. S1). The whole process was carried out at 25 °C.

2.4. Electrophoresis conditions

At the beginning of each working day, the capillary was consecutively rinsed with 0.2 M NaOH for 15 min, deionized water for 15 min and the running buffer for 15 min. Moreover, the capillary was flushed for 3 min between runs with running buffer. The support buffer was 12 mM $Na_2B_4O_7$–38 mM NaH_2PO_4 adjusted at pH 8.5. The voltage during separations was 10 kV and UV detection at 280 nm was employed. Samples were introduced into the capillary by hydrodynamic injection, where the sample vials were raised by 15 cm for 10 s.

2.5. Sample preparation

Blank urine samples were provided by a healthy male volunteer (27 years, 70 kg). After overnight urine was discharged, the participant drank 200 mL of water and then collected urine. The urine samples were respectively collected and stored in PTFE flasks at 4 °C before use. All the urine samples were centrifuged for 10 min at 4000 rpm and filtered with 0.45 μm polyether sulfone filters. Then, the samples were adjusted to pH 7.0 as donor solutions were prepared before SD-LLLME.

3. Results and discussion

3.1. Optimization of CE conditions

The running buffer was an important factor for the separation of FQs. The effect of several buffers with different concentrations, including 25 mM $Na_2B_4O_7$–25 mM NaH_2PO_4–25 mM H_3BO_3, 20 mM $Na_2B_4O_7$–60 mM NaH_2PO_4, 12 mM $Na_2B_4O_7$–38 mM NaH_2PO_4 and 25 mM Na_2HPO_4–25 mM NaH_2PO_4 solutions was tested. It was found that the running buffer of 12 mM $Na_2B_4O_7$–38 mM NaH_2PO_4 had better baseline separation. The effect of pH from 8.0 to 9.5 on separation was investigated. The running buffer with a pH value of 8.5 with 12 mM $Na_2B_4O_7$–38 mM

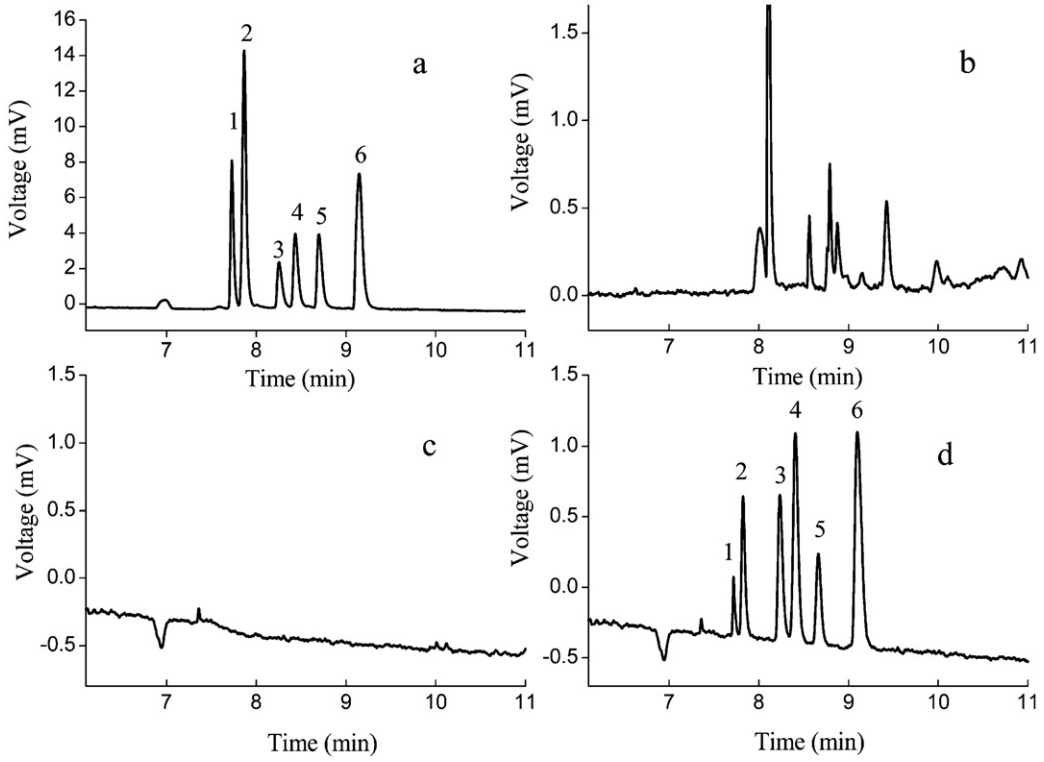

Fig. 2. Electropherograms of 100 μg mL^{-1} of the standard solution (a), the blank urine sample (b), the blank urine sample extract (c) and 100 μg L^{-1} of the spiked urine sample extract (d). Peak identification: (1) GAT, (2) LOM, (3) ENO, (4) CIP, (5) OFL, (6) PEF. CE conditions: running buffer, 12 mM $Na_2B_4O_7$–38 mM NaH_2PO_4 adjusted at pH 8.5; applied voltage, 10 kV; injection time, 10 s; UV detection, 280 nm. Extraction conditions: donor phase, 3.5 mL, pH 7.0; organic phase, 350 μL of DCM:toluene (1:1, v/v); extraction time, 40 min; stirring rate, 450 rpm; acceptor phase, 1 μL of 0.1 M NaOH and no salt addition.

NaH_2PO_4 was selected as the running buffer for separation. Then the effect of applied voltage in the range of 8–20 kV on the separation was tested. Only four FQs could be separated while the voltage higher than 10 kV. The maximum baseline separation was occurred at 10 kV which was chosen as the final separation voltage. The typical electropherogram of the standard mixture solution of six FQs was shown in Fig. 2a. Also we have given physico-chemical properties and the detailed retention data in Table 1.

3.2. Optimization of extraction conditions

3.2.1. Selection of organic solvent and its volume

Several organic solvents (*n*-octanol, toluene, xylene and ethyl acetate) in differing characteristics were investigated. Reduced chromatographic peak areas were found while they were used. DCM can be well used as organic solvent for the determination of FQs [15,26,27], but it cannot function well as middle phase both in SD-LLLME and HF-LLLME. For the SD-LLLME, DCM cannot float on the top of aqueous sample due to its higher density than water. And for the latter, with the low consumption and high volatility of DCM, it cannot remain in the pores of the polypropylene hollow fiber for more than a few seconds. However, we found that the mixture of dichloromethane and toluene (1:1, v/v) can float on the top of aqueous sample without using special equipment or other supporting material (Supplementary Fig. S2), although the density of mixture solvent was slightly greater than that of water (ρ, 1.089 g mL^{-1}), which was measured by hydrometer method. Merely based on the factor of density difference, it is hard to give a reasonable scientific explanation for the phenomenon mentioned above. So we have analyzed it synthetically and explain it as follows. The mixture solvent layer in the cylindrical vial was impacted by three forces [28]: upward floating force (F_f), downward gravity (F_g) and surface tension (σ). Under experimental conditions, the sum of σ and F_f of mixture (F_s) was bigger than F_g, so it would float on the water sur-

Table 1
Physico-chemical properties and detailed retention data of studied FQs.

Analytes	CAS no.	pK_a values[a]	MW[b]	t_m (s)[c]	R_s[d]	μ_{EOF} (cm^2 V^{-1} s^{-1})[e]	μ_a (cm^2 V^{-1} s^{-1})[f]	μ_e (cm^2 V^{-1} s^{-1})[g]
GAT	112811-59-3	5.94; 8.38	375.39	460.76 ± 2.66	–	4.93×10^{-4}	4.45×10^{-4}	-0.48×10^{-4}
LOM	98079-51-1	6.56; 8.47	351.35	468.92 ± 2.56	1.73	4.93×10^{-4}	4.37×10^{-4}	-0.56×10^{-4}
ENO	74011-58-8	6.00; 8.50	320.32	491.56 ± 3.57	3.21	4.93×10^{-4}	4.17×10^{-4}	-0.76×10^{-4}
CIP	85721-33-1	6.68; 8.63	331.34	502.56 ± 3.37	1.57	4.93×10^{-4}	4.08×10^{-4}	-0.85×10^{-4}
OFL	82419-36-1	6.67; 7.92	361.37	517.32 ± 4.86	2.21	4.93×10^{-4}	3.96×10^{-4}	-0.97×10^{-4}
PEF	70458-92-3	6.68; 7.83	333.36	545.62 ± 2.85	2.67	4.93×10^{-4}	3.76×10^{-4}	-1.17×10^{-4}

[a] Literature pK_a values of quinolones obtained from [23–25].
[b] Molecular weight.
[c] Migration time (means ± SD, $n = 3$).
[d] Resolution of two subsequent FQs.
[e] Electroosmotic flow mobility (EOF mobility).
[f] Apparent solute mobility.
[g] Effective solute mobility.

Table 2
Validation data of the proposed method for the determination of FQs.

Analytes	Calibration equation	Linear range (μg L^{-1})	Correlation coefficient (r^2)	LOD (μg L^{-1})	Recoveries (%) (RSD[a], %)	
					50 μg L^{-1}	100 μg L^{-1}
GAT	$y = 410.87x + 4.30$	40–1000	0.9963	31.48	82.9 (7.5)	100.9 (5.8)
LOM	$y = 685.25x + 8.57$	40–1000	0.9956	18.28	84.2 (8.3)	89.1 (7.2)
ENO	$y = 771.51x + 8.23$	40–1000	0.9962	15.06	102.8 (7.0)	103.2 (6.6)
CIP	$y = 1111.76x + 13.62$	40–1000	0.9919	10.78	87.5 (5.8)	100.2 (6.0)
OFL	$y = 1117.57x + 18.83$	40–1000	0.9913	23.24	83.0 (4.8)	104.9 (4.3)
PEF	$y = -633.68x + 50.39$	40–1000	0.9995	7.35	97.4 (4.6)	81.8 (7.8)

[a] RSD, relative standard deviation; $n = 3$.

face to form an organic layer instead of sinking into bottom of the bottle. Additionally, there was a significant increase in extraction efficiency for the mixture solvent compared to the single solvents. Thus, DCM:toluene (1:1, v/v) was selected as organic phase in the subsequent experiments.

Influence of the volume of the mixture was varied in the range of 300–450 μL in 50 μL intervals and the extraction efficiency was found to be better using a smaller volume. However, the organic layer formed by using 300 μL of DCM:toluene (1:1, v/v) was too thin to immerse the acceptor phase. As a compromise, 350 μL of organic phase was chosen in the following experiments.

3.2.2. Selection of stirring rate and extraction time

To evaluate the effect of sample stirring, working solutions were extracted with stirring rate varied in the range of 100–450 rpm. As expected, high stirring rate resulted in greater extraction efficiency. However, the acceptor phase was readily to drop when the stirring rate was higher than 450 rpm. As a result, stirring rate at 450 rpm was suitable in this work.

The extraction time was varied in the range of 10–50 min in 10 min intervals. The extraction efficiency increased with increasing extraction time, until the equilibrium between donor solution and acceptor phase was attained after extracting for 40 min. Longer extraction time was not recommended since the poor extraction efficiency and reproducibility was attained. So an extraction time of 40 min was selected.

3.2.3. Selection of pH of donor and acceptor phases

The pK_a values of FQs were generally from 7.24 to 8.70 for the ammonium form and from 5.66 to 6.81 for the carboxylic function [23–25]. In view of the pK_a values of FQs, the sample pH was studied within the range of 5.0–8.0. The highest extraction efficiency was achieved in neutral solution. Thus, donor solution was adjusted to pH 7.0 in the subsequent extractions.

In this study, NaOH solutions were selected as acceptor phase due to its better solubility of the FQs and compatibility with running buffer in CE. The pH of NaOH was varied to obtain better extraction efficiency. Finally, 0.1 M NaOH (pH 13) was used as acceptor phase since it provided highest extraction efficiency for the analytes.

3.2.4. Selection of NaCl concentration

For investigating the influence of salt addition on the extraction efficiency of SD-LLLME, NaCl at a concentration between 0 and 15 (w/v, %) was added to the sample solution. The results were shown that the peak areas decreased with the increment of NaCl concentration in the studied range. Thus, no salt addition was selected.

3.2.5. Volume ratio of donor to acceptor phase

In the present work, the effect of the volume ratio on extraction efficiency was evaluated by changing the volume of the acceptor phase in the range of 1.0, 1.5 and 2.0 μL while the volume of donor phase was kept constant at 3.5 mL. The results indicated that the highest extraction efficiency was obtained when 1 μL of NaOH was chosen as acceptor phase (i.e. donor/acceptor ratio of 3500:1), since the mass transfer took place more easily in a droplet with a smaller size [29].

3.3. Application to real samples

The influence of urine samples was investigated by injection of blank urine samples and blank urine samples extract. As shown in Fig. 2, there were some potential interfering peaks in the blank urine samples (Fig. 2b), however, these interfering components were eliminated after proposed here microextraction (Fig. 2c). Then, the urine samples were spiked at 100 μg L^{-1} with six FQs standards for the validation of the proposed technique. As illustrated in Fig. 2d, all of the analytes were selectively extracted and no interference was observed.

To further evaluate the practical applicability of the proposed technique, calibration standards at different concentrations in human urine for all FQs were used to construct the corresponding calibration curves. Results are shown in Table 2. It exhibited good correlation coefficients (0.9913–0.9995) for the concentration range of 40–1000 μg L^{-1} [30–34]. The LOD varied from 7.4 to 31.5 μg L^{-1} at a signal-to-noise (S/N) of 3. Good recoveries were obtained at two concentration levels (Table 2). The precision was determined after three consecutive extractions and the relative standard deviations (RSD) were 4.3–8.3%. The validation study shows that the developed method is accurate and robust.

3.4. Comparison of SD-LLLME with other methods

A comparison of the proposed technique with other related methods, which was used for the determination of FQs in urine samples, is given in Table 3. In comparison with other reported methods, the LODs of this technique are better than CE and MISPE-HPLC coupled of UV detector [6,8] and comparable with the data obtained by SPE-LC–MS [9] and MEPS-NACE-MS [10] although the

Table 3
Comparison of SD-LLLME-CE-UV with other methods for determination of FQs in urine samples.

Method	Linear range (μg L^{-1})	Correlation coefficients	RSD (%)	LOD (μg L^{-1})	Recovery (%)	Dilution	Reference
SPE-LC–MS	44–1000	>0.99	4.9–7.5	13–21	46.0–61.9	–	[5]
MISPE–HPLC-DAD	50–30,000	0.9993–0.9999	2.0–7.4	36–100	92.6–104.0	10 times	[6]
CE-UV	1000–120,000	0.995–0.996	3.41–1.25	200	96.8–102	2–5 times	[8]
MEPS-NACE-MS	12.5–500	0.994–0.998	3.4–5.9	6.3–10.6	71–109	5 times	[10]
SD-LLLME-CE-UV	40–1000	0.9913–0.9995	4.3–8.3	7.4–31.5	81.8–105.9	–	This study

sensitivity of MS is generally higher than that of UV detection. Additionally, the FQs can be selectively extracted and enriched, and no need for diluting the urine samples at a risk of further decreasing the analytical sensitivity of analytes. Despite this approach appears some shining point, it cannot be perfect due to its deficiency of limited stirring rate, which is also the weakness of common SD-LLLME procedures. Future work will focus on this to make perfection.

4. Conclusions

In this study, the mixture of DCM and toluene (1:1, v/v) was chosen as organic layer for the detection and quantification of FQs in urine samples using SD-LLLME combined with CE. This technique requires very little aqueous sample solution, organic solvent and acceptor solvent. The acceptor phase can be analyzed by CE system directly and no need for tedious steps such as phase separation, solvent evaporation and residual re-dissolution. Besides, good linearity, sensitivity, repeatabilities and relative recoveries were readily achievable in the actual application. All of the advantages mentioned above indicate that the proposed method is suitable for the determination of FQs in urine samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.11.040.

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