



SHORT COMMUNICATION

Highly sensitive trivalent copper chelate–luminol chemiluminescence system for capillary electrophoresis chiral separation and determination of ofloxacin enantiomers in urine samples



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Abstract A simple, fast and sensitive capillary electrophoresis (CE) strategy combined with chemiluminescence (CL) detection for analysis of ofloxacin (OF) enantiomers was established in the present work. Sulfonated β -cyclodextrin (β -CD) was used as the chiral additive being added into the running buffer of luminol–diperiodatocuprate (III) ($K_5[Cu(HIO_6)_2]$, DPC) chemiluminescence system. Under the optimum conditions, the proposed method was successfully applied to separation and analysis of OF enantiomers with the detection limits ($S/N=3$) of 8.0 nM and 7.0 nM for levofloxacin and dextroflaxacin, respectively. The linear ranges were both 0.010–100 μ M. The method was utilized for analyzing OF in urine; the results obtained were satisfactory and recoveries were 89.5–110.8%, which demonstrated the reliability of this method. This approach can also be further extended to analyze different commercial OF medicines.

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1. Introduction

Ofloxacin (OF), a quinolones antibiotic, has been widely used due to its broad-spectrum activity against both Gram-positive and Gram-negative organisms [1]. Some studies have demonstrated that the antibacterial activity of the levofloxacin is 8–128 times higher than that of dextroflaxacin and approximately 2 times higher than that of racemate [2]. Currently, the drug is either

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marketed as a racemic mixture, i.e., consisting of equal amounts of R- and S-enantiomers, or only the S-isomer. Therefore, studies on the separation of racemic ofloxacin and its application in pharmaceutical formulations are important.

Nowadays, several chiral extractants have been reported for the enantioselective analysis of OF. In the work of Fang et al. [3], cyclodextrin derivative was successfully applied for the separation of OF enantiomer. Li et al. [4] used natural biomacromolecules including BSA and DNA for enantioseparation of OF. Fu et al. [5] utilized functional Cu(II)-coordinated G-rich oligonucleotides for enantioselective separation of chiral OF. Among these chiral selectors, the CD was the most used in varieties of enantioselective analysis.

β -CD is the most popular chiral selector in the cyclodextrins [6,7]. However, the poor water solubility and the lack of functional groups limit the optimization of separation selectivity for enantiomers [2]. Chemical modification of β -CD could increase its solubility and the enantioseparation capacity. Recently, several studies about the use of charged β -CD derivatives [8–12] such as sulfated [9,10], hydroxypropyl [11] and methoxyethylammonium [12] β -CD as chiral selectors have been published. These CDs have been successfully employed in the enantioseparation of both basic and acidic chiral compounds. Sulfated β -CD is usually employed due to its relatively simple synthesis process than other β -CD derivatives. Therefore, in the present paper, sulfated β -CD was employed for the first time as a chiral selector in chiral separation of OF enantiomers.

CL detection was combined with CE separation, due to its distinguished sensitivity and inexpensive instrumentation. Diperio-datocuprate (III) ($K_5[Cu(HIO_6)_2]$, DPC), is a transition metal chelate at an unstable high oxidation state, just as our previous work [13,14] described in detail, and can react with luminol to generate a CL emission in a basic medium. Moreover, OF could greatly enhance this CL reaction to produce very strong CL signal. This CL system was utilized to develop a rapid and sensitive CE–CL method for the determination of OF. Using sulfonated β -CD as chiral additive, this method could be successfully applied for the separation and determination of OF enantiomers in urine samples.

2. Materials and methods

2.1. Chemicals and materials

Potassium persulfate ($K_2S_2O_8$), cupric sulfate ($CuSO_4 \cdot 5H_2O$), potassium hydroxide (KOH), and potassium periodate (KIO_4) were purchased from Kelong Chemical Reagent Plant (Chengdu, China). Luminol and ofloxacin were obtained from Sigma-Aldrich (St. Louis, USA). Levofloxacin was provided by the National Institutes for Food and Drug Control (Beijing, China). Dextro-floxacin was purchased from LKT Laboratories, Inc. (St. Paul, USA). Sulfated- β -CD ($M=1444$) was purchased from Binzhou Zhiyuan Bio-Technology Co., Ltd. (Shandong, China). Fused-silica capillary was provided by Yongnian Optical Fiber Co., Ltd. (Hebei, China). Phosphate buffer was prepared by dissolving 0.03 g of NaH_2PO_4 in 100 mL water, and the pH was adjusted with 1.0 M NaOH. The luminol stock solution at 10.0 mM was prepared by dissolving 0.18 g of luminol in 100 mL of 0.10 M NaOH solutions, and stored in the refrigerator at 4 °C. All solutions were prepared with ultra-pure water (18.2 M Ω) treated with ELGA PURELAB classic system (Veolia Water Solutions & Technologies Co., Ltd., London, UK). Early morning urine samples were collected from three healthy volunteers.

2.2. Synthesis of DPC

The DPC stock solution at 10.0 mM was prepared by oxidizing Cu (II) in a strong alkaline medium according to the previously reported method [15]. In brief, KIO_4 (0.23 g), $CuSO_4 \cdot 5H_2O$ (0.12 g), $K_2S_2O_8$ (0.14 g) and KOH (0.80 g) were mixed and dissolved in 30 mL of water. The solution was heated until boiling and kept at that temperature for about 20 min with constant stirring. The dark red product solution was then cooled to room temperature and diluted to 50 mL with water. The obtained DPC solution was stored in the refrigerator at 4 °C, and could be kept stable for no less than 5 months. The ultraviolet/visible absorbance spectrum of the chelate exhibited a broad band at 415 nm.

2.3. Apparatus and procedures

All the CE–CL experiments were performed on a home-assembled apparatus, which was constructed in a post-column flow-through detection mode [11]. Teflon tubes (0.80 mm I.D.) were used to connect all the components in the flow system. A fused-silica capillary (60 cm \times 75 μ m I.D.) was used for CE separation. In this study, the new capillaries were rinsed sequentially with 1.0 M NaOH solution for 30 min and water for another 30 min prior to the first use. Between two consecutive injections, the capillary was flushed sequentially with 0.10 M NaOH solution, water and RB for 2 min each. A 0.50-cm polyimide coating section at one tip of the capillary column was burned and removed. The burned tip of the capillary was directly inserted into an optical glass tube (3.0 cm \times 1.0 mm I.D.) acting as the CL detection window. The CL detection window was located above a photomultiplier (PMT) operated at -800 V for performing CL signal collection. Both the detection window and the PMT were sealed in a dark box to avoid interference from the external light.

The RB used for CE separation was 2.0 mM phosphate buffer (pH 4.0) containing 0.10 mM luminol and 3.0 mg/mL sulfated- β -CD, and the separation voltage was 12 kV. The solution of 0.10 mM DPC in 40 mM NaOH was siphoned into the detection window with an altitude difference of 25 cm, to trigger the CL reaction. After a stable baseline was obtained, the sample was loaded by gravity injection for 15 s at an altitude difference of 15 cm. The peak height of the electropherogram was applied as the quantitative parameter to evaluate the level of OF. Data acquisition and treatment were performed using an IFFM software package (Remex, Xi'an, China).

2.4. Urine sample preparation

A 500- μ L urine sample in a 3.0-mL centrifuge tube was mixed with 1.0 mL of acetonitrile and shaken vigorously for 2 min to precipitate proteins. After centrifuging at 10,000 rpm for 10 min, the supernatant was transferred into a 1.5-mL vial and dried with a gentle nitrogen stream. The residue was re-dissolved in 500 μ L of water. The processed samples were diluted with water if necessary after being filtered through a 0.22- μ m membrane filter.

3. Results and discussion

3.1. Kinetic characteristic of OF–luminol–DPC CL reaction

The preliminary experiment demonstrated that luminol could be oxidized by DPC to produce CL emission in alkaline solution, and

this CL emission could be greatly enhanced in the reaction in the presence of OF. The kinetic characteristics of both DPC–luminol–OF and DPC–luminol CL reactions were tested by a static mode, and the typical response curves (CL intensity versus times) are shown in Fig. 1. It was found that the CL emission only took the maximum of 0.3 s after DPC was injected into the mixture of luminol and OF. Such a rapid kinetic characteristic for DPC–luminol–OF CL reaction was very helpful in obtaining a high sensitivity since the contacting time of DPC and the effluent from the capillary was very short in the flow-through detection interface.

3.2. Chiral separation of OFs

CDs have been successfully utilized as chiral selectors in CE for enantiomeric separations of chiral drugs and other compounds. In this method, β -CD, tosyl- β -CD and sulfated- β -CD were tested for the enantiomeric separation of OFs. β -CD and tosyl- β -CD showed limited solubility and slight interaction with the analytes. The electropherograms obtained from the three chiral selectors are shown in Fig. 2. A satisfactory chiral recognition was achieved when sulfated- β -CD was chosen as the chiral selector. The concentration of sulfated- β -CD was a key factor influencing the chiral resolution of OFs. The highest signal intensity and the best peak shape were obtained when the concentration of sulfated- β -CD was 3.0 mg/mL.

3.3. Condition optimization

To obtain a good analytical performance, we investigated the dependence of the column efficiency and the relative CL intensity on the RB conditions. The results showed that a basic RB led to a very poor chiral separation when sulfated- β -CD was used as the chiral selector. Phosphate buffer at pH 4.0 (2.0 mM) was finally chosen as the optimal RB because it provided satisfactory chiral recognition and an acceptable relative CL signal intensity. The influence of the luminol concentration in RB was investigated in the range of 0.02–0.50 mM, and the optimum concentration for the CL reaction was found to be 0.10 mM.

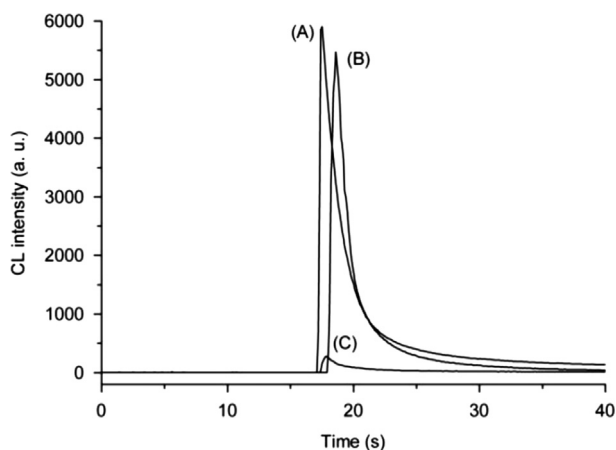


Fig. 1 Kinetic curves for (A and B) luminol–DPC–OF and (C) luminol–DPC CL systems. 100 μ L DPC solution (5.0 μ M) was injected into a mixture of 0.50 mL of 1.0 μ M luminol (in 40 mM NaOH), 10 μ L of 3.0 mg/mL sulfated- β -CD and 1.0 mL of 5.0 μ M (A) levofloxacin or (B) dextroflouxacin. (C) 100 μ L DPC solution (5.0 μ M) was injected into a mixture of 0.50 mL of 1.0 μ M luminol (in 40 mM NaOH), 10 μ L of 3.0 mg/mL sulfated- β -CD and 1.0 mL of water.

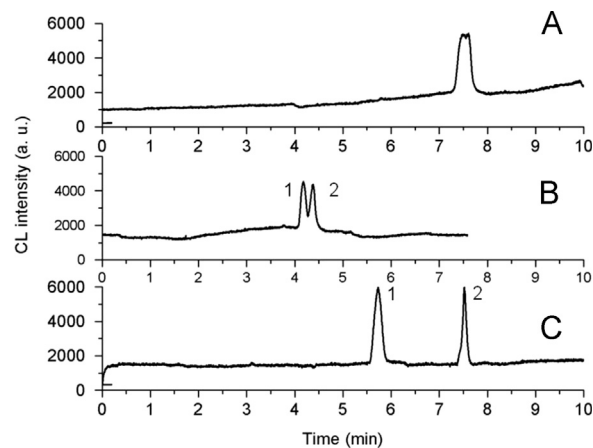


Fig. 2 Electropherograms for analysis of 40 μ M OF. Running buffer: 2.0 mM phosphate buffer (pH 4.0) containing 0.10 mM luminol and 3.0 mg/mL β -CD (A), or 3.0 mg/mL tosyl- β -CD (B), or 3.0 mg/mL sulfated- β -CD (C). The solution of 0.10 mM DPC in 40 mM NaOH was siphoned into the detection window with an altitude difference of 25 cm. 1=dextroflouxacin, 2=levofloxacin.

DPC was used as an efficient oxidant in this CL reaction, and showed a great effect on the generation of CL signal. The effect of DPC concentration on the CL signal was studied in the range of 0.025–0.40 mM. As shown in Fig. 3A, the CL signal increased with the concentration of DPC, and reached the maximum when DPC was 0.10 mM. The decreased CL intensity at higher DPC concentration could be attributed to the self-absorption of the DPC solution, since DPC showed a peak absorbance at 415 nm, which was very near the maximum emission wavelength of luminol (425 nm). NaOH provided the required alkaline environment in this CL system. As shown in Fig. 3B, the effect of the NaOH concentration on the relative CL intensity was investigated in the range of 10–60 mM. The results indicated that the maximal relative CL intensity was reached when the NaOH concentration was 40 mM; however, higher concentration of NaOH also decreased the CL signal. Thus, 40 mM was chosen as the optimal NaOH concentration.

In order to avoid the electrical discrimination resulting from the electromigration injection, the sample was loaded into the capillary column by an altitude difference. The increased loading time and height difference led to a high CL signal owing to a large sample loading amount, which also resulted in a broadened peak and a decreased resolution. After careful investigation, the loading time and the height difference were chosen to be 15 s and 20 cm, respectively.

3.4. Analytical performance

Under the optimal conditions, a series of levofloxacin and dextroflouxacin standard samples at different concentrations were assayed using the proposed CE–CL method. The linear ranges were both 0.010–100 μ M, with the detection limits ($S/N=3$) of 8.0 nM and 7.0 nM for levofloxacin and dextroflouxacin, respectively. The correlation coefficients for levofloxacin and dextroflouxacin were 0.9912 and 0.9917, respectively. The detection limits of levofloxacin and dextroflouxacin were about 100 times lower than those obtained with the previously reported HPLC combined with UV–vis absorbance detection [16]. The whole chiral separation process could be completed within 9 min with the

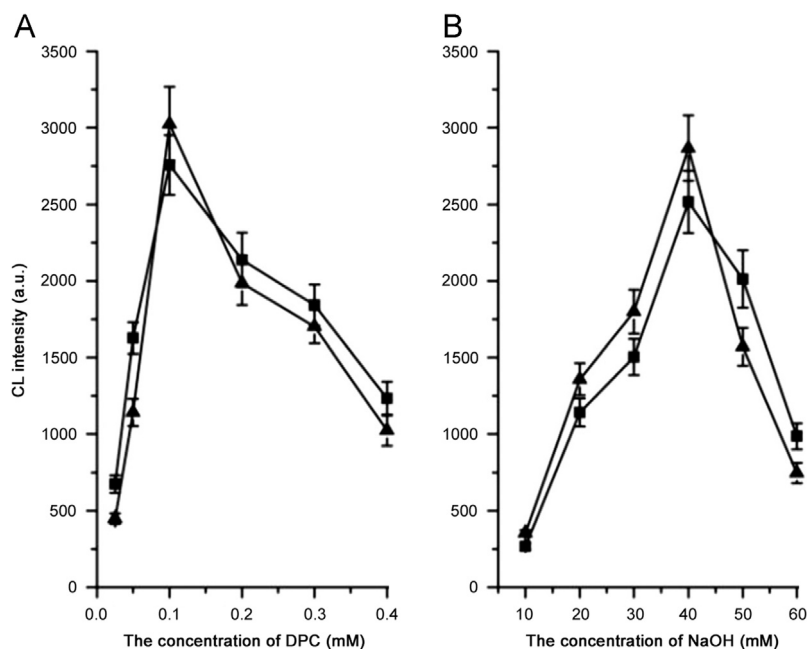


Fig. 3 Effects of the concentration of (A) DPC and (B) NaOH on the CL signal from (■) levofloxacin and (▲) dextrofloxacine at 10 μM . All other conditions were the selected optimal condition ($n=5$).

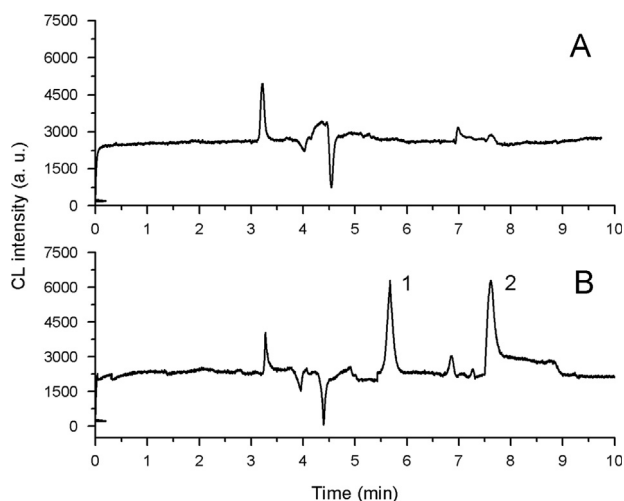


Fig. 4 Electropherograms for analysis of (A) blank urine sample and (B) urine sample spiked with OFs at 40 μM . All the conditions were the selected optimal conditions. 1=dextrofloxacine, 2=levofloxacin.

theoretical plate numbers of 18,600 and 14,800 for dextrofloxacine and levofloxacin, respectively. The reproducibility was assessed by intra- and inter-day RSDs for 12 replicate determinations of levofloxacin and dextrofloxacine standard samples at 10 μM . The intra- and inter-day RSDs for CL signals were 6.2% and 7.1%, and those for migration times were 4.8% and 5.2%, respectively.

3.5. Determination of OF in human urine

The proposed strategy was also utilized for the detection of OF in three human urine samples to evaluate its potential application value in pharmacokinetic investigation. The research involving samples from human has been approved of by the local research ethics committee. The electropherograms of the urine samples are

Table 1 The results of urine analysis and the recovery studies of OF ($n=5$).

Samples	Added (μM)	Found (μM)	Recovery (%)
Urine 1	20.0	18.5 ± 0.8	92.5
	40.0	41.5 ± 2.3	103.8
	70.0	74.4 ± 5.6	106.3
Urine 2	20.0	17.9 ± 0.9	89.5
	40.0	44.3 ± 2.8	110.8
	70.0	72.2 ± 6.5	103.1
Urine 3	20.0	20.5 ± 1.1	102.5
	40.0	38.2 ± 2.3	95.5
	70.0	69.7 ± 5.8	99.6

shown in Fig. 4. The three urine samples were also spiked with OF standards at different concentrations and assayed using the proposed method. Recoveries from 89.5% to 110.8% were obtained as shown in Table 1. To assess the repeatability, five consecutive determinations of OF in urine samples were performed. The RSDs for the migration time, the peak heights and recoveries were less than 6.4%, 7.3% and 8.3%, respectively.

4. Conclusion

OF enantiomers were successfully separated by the established CE-CL strategy. The presence of sulfated- β -CD in the running buffer has played an important role in enhancing resolution between enantiomers. DPC, a transition metal chelate at unstable high oxidation state, was synthesized in a strong basic solution, and acted as the oxidant in this novel CL reaction. The method was applied in spiked urine samples. Sulfated- β -CD as an additive of chiral separation provides a new approach for the separation of

chiral drugs which are difficult to be separated under the common electrophoresis conditions. Finally, the proposed method could be applied to analyze commercial ofloxacin medicines. This study exhibits the potential for the separation and determination of other chiral medicines by the CE–CL method.

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References

- [1] I. Morrissey, K. Hoshino, K. Sato, et al., Mechanism of differential activities of ofloxacin enantiomers, *Antimicrob. Agents Chemother.* 40 (1996) 1775–1784.
- [2] N. Rosales-Conrado, M.E. Leon-Gonzalez, A. Rocco, et al., Enantiomeric separation of ofloxacin by nano-liquid chromatography using a sulfated- β -cyclodextrin as a chiral selector in the mobile phase, *Curr. Anal. Chem.* 6 (2010) 209–216.
- [3] Z.L. Fang, Z.Y. Guo, Q. Qin, et al., Semi-preparative enantiomeric separation of ofloxacin by HPLC, *J. Chromatogr. Sci.* 51 (2013) 133–137.
- [4] W. Li, Y.L. Li, Y. Fu, et al., Enantioseparation of chiral ofloxacin using biomacromolecules, *Korean J. Chem. Eng.* 30 (2013) 1448–1453.
- [5] Y. Fu, X.L. Duan, X.F. Chen, et al., Enantioselective separation of chiral ofloxacin using functional Cu(II)-coordinated G-rich oligonucleotides, *RSC Adv.* 4 (2014) 1329–1333.
- [6] H. Nishi, S. Terabe, Optical resolution of drugs by capillary electrophoretic techniques, *J. Chromatogr. A* 694 (1995) 245–276.
- [7] G. Tóth, R. Mohácsi, Á. Rácz, et al., Equilibrium and structural characterization of ofloxacin–cyclodextrin complexation, *J. Incl. Phenom. Macrocycl. Chem.* 77 (2013) 291–300.
- [8] G. Gübitz, M.G. Schmid, Chiral separation by capillary electromigration techniques, *J. Chromatogr. A* 1204 (2008) 140–156.
- [9] L. Asensi-Bernardi, L. Escuder-Gilabert, Y. Martín-Biosca, et al., Modeling the chiral resolution ability of highly sulfated β -cyclodextrin for basic compounds in electrokinetic chromatography, *J. Chromatogr. A* 1308 (2013) 152–160.
- [10] I. Fejős, Y. He, G. Völgyi, et al., Tapentadol enantiomers: synthesis, physico-chemical characterization and cyclodextrin interactions, *J. Pharm. Biomed. Anal.* 88 (2014) 594–601.
- [11] Y. Cui, X.W. Ma, M. Zhao, et al., Combined use of ionic liquid and hydroxypropyl- β -cyclodextrin for the enantioseparation of ten drugs by capillary electrophoresis, *Chirality* 25 (2013) 409–414.
- [12] S.Y. Wang, Y. Dai, J.H. Wu, et al., Methoxyethylammonium monosubstituted β -cyclodextrin as the chiral selector for enantioseparation in capillary electrophoresis, *J. Chromatogr. A* 1277 (2013) 84–92.
- [13] Z.F. Fu, Z.Y. Li, H.Y. Xie, et al., Highly sensitive trivalent copper chelate– H_2O_2 system for CE-chemiluminescent detection of luminol-type compounds, *Electrophoresis* 31 (2010) 3342–3345.
- [14] T. Li, Z.R. Wang, H.Y. Xie, et al., Highly sensitive trivalent copper chelate–luminol chemiluminescence system for capillary electrophoresis detection of epinephrine in the urine of smoker, *J. Chromatogr. B* 911 (2012) 1–5.
- [15] A. Balikungeri, M. Pelletier, D. Monnier, Contribution to the study of the complexes bis(dihydrogen tellurato)cuprate(III) and argentate(III), bis(hydrogen periodato)cuprate(III) and argentate(III), *Inorg. Chim. Acta* 22 (1977) 7–14.
- [16] H.Y. Yan, K.H. Row, Rapid chiral separation and impurity determination of levofloxacin by ligand-exchange chromatography, *Anal. Chim. Acta* 584 (2007) 160–165.