High performance liquid chromatography coupled with resonance Rayleigh scattering for the detection of three fluoroquinolones and mechanism study

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The highlighting of the paper:
- The method integrates the high sensitivity of RRS and the high selectivity of HPLC.
- The method has been applied to determine FQs in human urine sample and water sample.
- This work can provide research foundations for improvement of method and apparatus.
- Quantum chemistry calculation and FTIR were used to discuss the reaction mechanism.
- New reaction mechanism was proposed and validated in this paper.

Abstract
A reliable and versatile high performance liquid chromatography coupled with resonance Rayleigh scattering method was established for the determination of three fluoroquinolones, including levofloxacin, norfloxacin and enrofloxacin in water sample and human urine sample. In pH 4.4–4.6 Britton–Robinson buffer medium, the fluoroquinolones separated by high performance liquid chromatography could react with erythrosine to form 1:1 ion-association complexes, which could make contributions to the great enhancement of RRS. The resonance Rayleigh scattering signal was recorded at \( \lambda_{ex} = \lambda_{em} = 330 \) nm. The resonance Rayleigh scattering spectral characteristics of the drugs and the experimental conditions such as pH, detection wavelength, erythrosine concentration, flow rate, the length of reaction tube were studied. Quantum chemistry calculation, Fourier transform infrared spectroscopy and absorption spectroscopy were used to discuss the reaction mechanism. The recoveries of samples added standard ranged from 97.53% to 102.00%, and the relative standard deviation was below 4.64%. The limit of detection (S/N = 3) of 0.05–0.12 \( \mu g \) mL\(^{-1}\) was reached, and the linear regression coefficients were all above 0.999. The proposed method was proved as a simple, low cost and high sensitivity method.

Keywords:
High performance liquid chromatography
Resonance Rayleigh scattering
Fluoroquinolones
Erythrosine

Introduction
The fluoroquinolones (FQs) are bactericidal and act principally by inhibition of bacterial DNA-gyrase [1]. Due to their broad-spectrum activity against both Gram-positive and Gram-negative bacteria [2], they are currently in wide usage for the clinical treatment of severe systemic infections, including urethral infections, respiratory infections, soft tissue infections and so on [3]. Norfloxacin (NOR), levofloxacin (LEV) and enrofloxacin (ENRO) are the third generation members of fluoroquinolone antibiotics, which have wider antimicrobial spectra, more significant
changes in their antibacterial potency [3]. In particular, enrofloxa-
cin as the first fluoroquinolone developed for veterinary applica-
tion, was widely used as antimicrobial agents in animal
husbandry and aquaculture. Furthermore, enrofloxacin is a syn-
thetic antibacterial agent, so it is not official in any pharmacopoeia
[4]. In recent years, A variety of analytical techniques such as
spectrofluorometry [5], Resonance Rayleigh scattering (RRS) [3,6],
flow injection chemiluminescence [7], surface plasmon resonance
(SPR) [8], capillary electrophoretic-fluorescence [9], enzyme linked
immunosorbtent assay (ELISA) [10], HPLC–MS, HPLC–UV, HPLC–
FLD, HPLC–RRS [11–14] have been reported for the analysis of
the FQs.

Resonance Rayleigh scattering (RRS) as a newly analytical tech-
nique has received extensive attention and application, and has
been applied to many fields, such as biological macromolecules
[15], pharmaceuticals [16], environment [17], natural products
[18] and some physicochemical parameters [19]. Nowadays, the
RRS technique is gradually being used to study system reaction
mechanisms [20]. High performance liquid chromatography–reso-
nance Rayleigh scattering (HPLC–RRS) method integrates the high
sensitivity of RRS and the high selectivity of HPLC, which has been
applied to study and determination amino-glycosides [21], fluoro-
quinolones [13], four tetracycline antibiotics [22], local anesthetics
[23], amino acids [24], proteins [25] and so on.

In the present paper, HPLC–RRS analysis technique has been
applied and validated in the detection of norfloxacin, levofloxacin
and enrofloxacin in water sample and urine sample. A series of
experimental parameters of HPLC separation and RRS detection
were investigated to obtain accurate assay and a highly sensitive.
Quantum chemistry calculations were utilized to discuss the inter-
action mechanism and binding mode of the system. The results
indicated that norfloxacin, levofloxacin and enrofloxacin could
form an ion-association complex with erythrosine by electrostatic
attraction and hydrogen bonding, and then give rise to the signifi-
cant enhancement of scattering.

Experimental

Apparatus

An Agilent 1100 liquid chromatography (Agilent Technologies,
USA) was consisted of G1322A online degasser, G1311A pump,
G1316A column oven, G1321A fluorescence detector. A PCX-BT
post-column derivatization instrument was from Tian Mei Da Sci-
entific Instruments Co., Ltd (Shenyang, China). The RRS intensities
and spectra were recorded on a Hitachi F-2500 spectrofluoropho-
tomer (Hitachi Ltd., Tokyo, Japan). Scanning electron microscopy
(SEM, S-4800, Hitachi, Japan) was used to observed the shape and
size of particles. Fourier transform infrared spectrometer (FTIR, PE
Co., U.S.A.) was used to get the infrared Spectrums. Double distilled
water was prepared by a Millipore SZ-93 system (Shanghai Yarong
Biochemical Apparatus, Shanghai, China).

Chemicals and reagents

Norfloxacin and enrofloxacin were purchased from National
Institute for the Control Pharmaceutical and Biological Products
(Beijing, China). Levofloxacin and erythrosine sodium (Ery) were
provided by Aladdin Chemical Reagents Ltd (Beijing, China)
HPLC-grade methanol and isopropanol were purchased from Ker-
mel (Tianjin, China). BR buffer solution of different pH was used
to control the acidity of the aqueous medium. All of Analytical
reagent-grade NaOH, NaAc, H₃PO₄ and HAc were supplied by
Chemistry Reagent Factory (Chengdu, China).
Preparation of standards and sample pretreatment

Preparation of standards
Norfloxacin, levofloxacin, and enrofloxacin were weighed and dissolved in some hydrochloric acid (0.1 mol L$^{-1}$), then diluted it to the mark with water to prepare a stock solution of 1.0 mg mL$^{-1}$. All the solutions were stored at 0–4°C in darkness. The daily standard working solutions of different concentrations were obtained by serial diluted the stock solutions. Double distilled water was used throughout. All reagents were filtered through a 0.22 μm pore-size filter membrane (Millipore, Bedford, MA, USA).

Water samples
The water samples were obtained from a fishpond located around the city of beibei, spiked with a certain amounts of norfloxacin, levofloxacin and enrofloxacin, and then filtered through the 0.22 μm Nylon membrane filters.

Urine samples
Human urine samples were collected from a healthy volunteer, and the analysis was conducted immediately after the sample collection. 400 μL of acetonitrile was added to 200 μL of human urine to precipitate protein. The samples was vortex mixed for 30 s and centrifuged for 15 min at 8000 rpm min$^{-1}$, transferred the clear supernatant to a clean centrifuge tube, evaporated the organic phase in a vacuum drying oven at 45°C and reconstituted with redistilled water, then filtered through the 0.22 μm Nylon membrane filters.

HPLC–RRS system
The HPLC–RRS detection system was schematically shown in Fig. 1. The protonation of drugs separated by HPLC was strengthened in BR solution at interface 1, then protonated drugs reacted with Ery to form complexes at heated reacting tube whose length could be selected according to the need, finally complexes were detected at FLD detector.

Table 1
Structures of the investigated fluoroquinolones.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>R$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin</td>
<td>O</td>
<td>O</td>
<td></td>
<td>H</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>H</td>
<td></td>
<td>H</td>
<td>C$_2$H$_5$</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>C$_2$H$_5$</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>

Fig. 2. RRS spectra: (1) LEV; (2) NOR; (3) ENRO; (4) Ery; (5) LEV–Ery; (6) NOR–Ery; (7) ENRO–Ery; (a) SEM image of Ery; (b) SEM image of ENRO–Ery. FQs concentration: 1 μg mL$^{-1}$. Ery concentration: 3.0 × 10$^{-5}$ mol L$^{-1}$. BR: pH 4.5.

Fig. 3. Effect of acidity: FQs concentration: 1 μg mL$^{-1}$. Ery concentration: 3.0 × 10$^{-5}$ mol L$^{-1}$.

Fig. 4. The effect of the flow rate of the HPLC mobile phase was studied.

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methanol (76/24, v/v), with isocratic elution at 0.6 mL min\(^{-1}\). The intensities of RRS were measured at \(\lambda_{ex} = \lambda_{em} = 330\) nm.

**Results and discussion**

**Selection of the detection wavelength**

Place 1.0 mL of BR buffer solution, 1.0 mL of Ery and suitable amounts of fluoroquinolones solution into a 10.0 mL calibrated flask in turns, then diluted it to the mark with water and mixed thoroughly. The RRS spectra was recorded by synchronous scanning at \(\lambda_{ex} = \lambda_{em}\) on the Hitachi F-2500 spectrofluorophotometer. A dramatic enhancement and the maximum of the RRS intensity at 330 nm could be seen from Fig. 2. Thus, a series of RRS wavelength such as 290, 310, 330, 350, 370 and 565 nm were investigated in HPLC to obtain the optimal detection wavelength. The results showed us that all the peak areas reached the maximum at 330 nm.

**Effect of acidity**

The acidity played a vital role in the interaction between FQs and Ery. Thus, different pH values of BR buffer in the range of

![Black: Mulliken atomic charges, Red: Natural Population Analysis](image)

Fig. 5. The charge distribution of ENRO: the black color was by Mulliken atomic charges method, and the red color was by natural population analysis method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![A, B, C](image)

Fig. 6. The optimized stability of systems from B3LYP/6-31G calculation: (A) Ery; (B) ENRO; (C) ENRO–Ery.
results indicated that the suitable Ery concentration was $5.0 \times 10^{-4}$ mol L$^{-1}$. The RRS intensity decreased with the decreasing of Ery concentration, but the increase of Ery concentration could lead to the increase of noise. Therefore, $5.0 \times 10^{-4}$ mol L$^{-1}$ was selected as the optimum concentration of Ery.

**Effect of the flow rate**

The flow rates of the HPLC mobile phase have a very important effect on the chromatographic separation and the RRS intensity. The flow rates of the HPLC mobile phase from 0.5 to 0.7 mL min$^{-1}$ were optimized. As showed in Fig. 4, when the flow rate was lower than 0.5 mL min$^{-1}$, the retention time would be prolonged and the sensitivity decreased. On the other hand, when the flow rate was 0.7 mL min$^{-1}$, the column pressure increased and the sensitivity also decreased. Therefore, 0.6 mL min$^{-1}$ was selected as the optimal flow rate in this work.

A systematic multifactorial experimental design has been applied in this work. In consideration of the greatest effect on this reaction system, this work kept the reaction conditions at pH 4.5 by adjusting the pH of BR buffer solution, and the flow rates of Ery and BR were also optimized from 0.1 to 0.6 mL min$^{-1}$. When Ery was 0.2 mL min$^{-1}$ and BR (pH 11.0) was 0.4 mL min$^{-1}$, the $I_0$ was the minimum and the $I_{RRS}$ reached the maximum.

**Effect of the length of reaction tube**

As the reaction place for the combination of drugs and Ery, the length of the reaction tube and the reaction time were closely related to the complete degree of reaction. Given this, the reaction tube lengths in the range of 100–400 cm were studied, and the intensity of RRS sharply enhanced with the increase of the length up to 300 cm. However, the increase of dead volume which caused by reaction tube resulted in decreased signal. Hence, 300 cm was selected for this experiment.

**Reaction mechanism of the system**

Form Table 1, it is easy to see that the investigated fluoroquinolones have the same parent structure and only have different substituent groups. ENRO was opted as the example to discuss the mechanism of the system. In pH 4.5 weak acid medium, Ery as a binary weak acid (H$_2$L) whose $pK_a$ was 3.6, would dissociate as HL$^{-}$ and L$^{2-}$ types [26,27]. HL$^{-}$ was the main species of erythrosine, but the main type which could react with ENRO was L$^{2-}$ by

### Table 2

<table>
<thead>
<tr>
<th>FQs</th>
<th>Linear regression Equation ($c$, µg mL$^{-1}$)</th>
<th>Linear range (µg mL$^{-1}$)</th>
<th>Correlation Coefficient ($r$)</th>
<th>LOD (µg mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEV</td>
<td>$y = 88.87 + 105.1x$</td>
<td>0.200–15.0</td>
<td>0.9995</td>
<td>0.21</td>
</tr>
<tr>
<td>NOR</td>
<td>$y = 155.4 + 153.6x$</td>
<td>0.250–16.3</td>
<td>0.9992</td>
<td>0.18</td>
</tr>
<tr>
<td>ENRO</td>
<td>$y = 53.86 + 203.1x$</td>
<td>0.180–19.2</td>
<td>0.9993</td>
<td>0.12</td>
</tr>
</tbody>
</table>

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using quantum chemistry methods and Fourier transform infrared (FTIR) spectroscopy.

**Quantum chemistry methods**

In the present work, full geometry optimizations of the studied systems were performed using the B3LYP functional with 6-31G basis set (the pseudo potential basis sets lanl2dz for iodine atoms). The stability of the optimized equilibrium geometry was checked by a frequency calculation. Meanwhile, the electronic and thermal Free Energies were also provided for reference. All calculations presented in this work were carried out by the Gaussian 09 program package. Two density function theory methods which were Mulliken atomic charges and natural population analysis were used to calculate the charge distribution of ENRO. The results showed that the charge density of oxygen atom of –OH was the highest (Fig. 5). Because of the strong attracting electron group (–I) near the hydroxyl on xanthene group of Ery, which decreased the electron density of oxygen on hydroxyl, the hydroxyl on xanthene group was easier to dissociate than –COOH on phenyl. It was more likely to react with the –COOH of ENRO by electrostatic attraction and hydrogen bonds (Fig. 6). What is more, the stabilization energy could reach $/C_0 \text{kJ mol}^{-1}$ after the reaction of Ery and ENRO.

**Fourier Transform infrared spectroscopy (FTIR)**

The Fourier transform infrared Spectrum (FTIR) of ENRO, Ery and ENRO–Ery (Fig. 7) also demonstrated that, the reaction site of ENRO–Ery was at the hydroxyl on xanthene group of Ery and the carboxyl of ENRO. From Fig. 6C, we could see the $/C_{\text{C-N}}$ at 1079 cm$^{-1}$ of ENRO and the $/C_{\text{C=O}}$ at 470 cm$^{-1}$ of Ery. However, the absorption bands at 1730 cm$^{-1}$ which could be assigned to the $/C_{\text{C=O}}$ on –COOH of ENRO, vanished after ENRO react with Ery. The peaks at 3445 cm$^{-1}$ due to –OH stretching of the hydroxyl groups could be seen at all the three figures.

### The reasons for RRS enhancement

Firstly, RRS is a scattering–absorbing–re-scattering process, When the wavelength of Rayleigh scattering is located or is close to the molecular absorption band, the scattering can absorb the light energy and to produce a re-scattering process by resonance (Fig. 8) [28]. So the resonance enhanced effect is an important reason for scattering enhancement of this system. Second, the diameter and shape of the formed aggregates were measured by the SEM. The SEM images showed that the single Ery molecule (a) could hardly be found and the ENRO–Ery (b) complex had aggregated (be inserted in Fig. 2), that was a significant factor to the enhancement of RRS intensity [29]. What is more, the remarkable increase of the mean polarizability and hydrophobicity after the reaction were also profitable to the enhancement of scattering intensity [3].

### Method validation and application

#### Method validation

Under the optimum conditions, a series of mixture solutions containing different concentrations of LEV, NOR, and ENRO were analyzed. All the analytical parameters are presented in Table 2.

### Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>FQs</th>
<th>Initial (μg mL$^{-1}$)</th>
<th>Added (μg mL$^{-1}$)</th>
<th>Found (μg mL$^{-1}$)</th>
<th>Recovery (%)</th>
<th>RSD% (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine sample</td>
<td>LEV</td>
<td>ND</td>
<td>3.00</td>
<td>2.93</td>
<td>97.53</td>
<td>4.53</td>
</tr>
<tr>
<td></td>
<td>LEV</td>
<td>ND</td>
<td>9.00</td>
<td>9.10</td>
<td>101.09</td>
<td>3.61</td>
</tr>
<tr>
<td></td>
<td>NOR</td>
<td>ND</td>
<td>3.00</td>
<td>2.95</td>
<td>98.20</td>
<td>3.13</td>
</tr>
<tr>
<td></td>
<td>NOR</td>
<td>ND</td>
<td>9.00</td>
<td>8.97</td>
<td>99.71</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>ENRO</td>
<td>ND</td>
<td>3.00</td>
<td>3.05</td>
<td>101.67</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>ENRO</td>
<td>ND</td>
<td>9.00</td>
<td>9.07</td>
<td>100.82</td>
<td>2.08</td>
</tr>
<tr>
<td>Water sample</td>
<td>LEV</td>
<td>ND</td>
<td>3.00</td>
<td>3.06</td>
<td>102.00</td>
<td>4.64</td>
</tr>
<tr>
<td></td>
<td>LEV</td>
<td>ND</td>
<td>9.00</td>
<td>8.86</td>
<td>98.49</td>
<td>2.09</td>
</tr>
<tr>
<td></td>
<td>NOR</td>
<td>ND</td>
<td>3.00</td>
<td>2.99</td>
<td>99.53</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>NOR</td>
<td>ND</td>
<td>9.00</td>
<td>8.95</td>
<td>99.44</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>ENRO</td>
<td>ND</td>
<td>3.00</td>
<td>3.01</td>
<td>100.27</td>
<td>3.84</td>
</tr>
<tr>
<td></td>
<td>ENRO</td>
<td>ND</td>
<td>9.00</td>
<td>9.00</td>
<td>100.00</td>
<td>2.15</td>
</tr>
</tbody>
</table>

ND: not detected.
The rendering of calibration curve uses the peak area of FQs (y) and the concentration of FQs (x, μg mL⁻¹). The limit of detection (LOD) was obtained at a signal-to-noise ratio (S/N) of 3. The assay demonstrated satisfactory linear relationship as well.

The HPLC–RRS method for the determination of FQs has high selectivity, high sensitivity, reliability, accuracy and rational recovery. On account of the poor selectivity, some methods such as spectrofluorometry, resonance Rayleigh scattering, surface plasmon resonance, enzyme linked immunosorbent assay are limited and interference problems may sometimes arise. On the other hand, some methods with high sensitivity have more or less inadequacies in accuracy and recovery (shown in Table 3). Furthermore, the method without complex pretreatment, and all reagents used in this paper are almost non-toxic and inexpensive.

Method application

The proposed analysis method was applied to detect FQs in human urine and fishpond water (Fig. 9), and the samples were handled as described above. Urine samples and water samples spiked with FQs were undertaken to calculate the recoveries, and the results are summarized in Table 4. From the results we could see that the recoveries were between 97.53% and 102.00%, and RSDs were ranged from 1.32% to 4.64%. So the method was dependable.

Conclusions

In summary, HPLC–RRS has been confirmed as a reliable and versatile method for the determination of LEV, NOR and NOR in human urine sample and water sample. The method showed a good linear, recovery and precision. The repeatability was much better than that of the RRS method itself. The optimization process of the method can provide research foundations for the improvement of method and apparatus. For example, the future work should focus on shortening the pipe to reduce the analysis time, and selecting a suitable flow-through cell to improve the HPLC–RRS sensitivity.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.saa.2014.10.004.

Reference