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Complexation-based spectrofluorimetric method for besifloxacin determination: Combined experimental and computational insights

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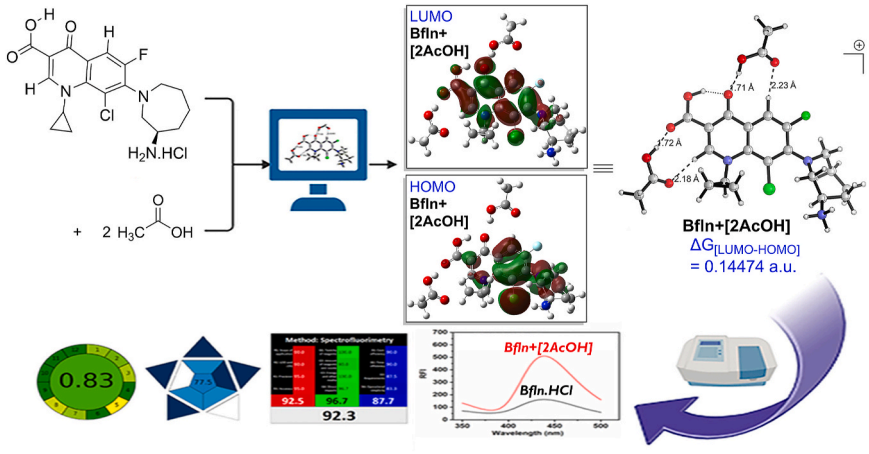
HIGHLIGHTS

- A novel fluorescence method for the estimation of besifloxacin was proposed.
- The DFT calculations were applied to confirm the fluorescence enhancement mechanism.
- Greenness, blueness and whiteness of the proposed fluorescent method were evaluated.
- The method was applied to quantify besifloxacin in eye drops and artificial aqueous humor.

GRAPHICAL ABSTRACT



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ABSTRACT

Fluorescence-based analytical methods offer high sensitivity, cost-effectiveness, and simplicity for pharmaceutical analysis. Besifloxacin hydrochloride (Bfln.HCl), a fluoroquinolone antibiotic used to treat eye infections, exhibits native fluorescence that can be enhanced in acidic media. However, the mechanism of this enhancement has not been fully explored. To address this, we developed a novel spectrofluorimetric method that enhances Bfln.HCl fluorescence using acetate buffer of pH 4.0. A 10 nm redshift in the excitation wavelength and a

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Aqueous humor
Ophthalmic analysis
Green chemistry

significant increase in fluorescence intensity at 440 nm were observed upon the addition of acetate buffer. Density Functional Theory (DFT) calculations revealed that the fluorescence enhancement results from the formation of 1:2 cyclic complexes between $Bfln^+$ and acetic acid (AcOH). Thermodynamic analysis indicated that the **$Bfln^+$/[2AcOH]** complex was the most stable, exhibiting the lowest HOMO-LUMO energy gap, consistent with the observed fluorescence enhancement. The method demonstrated high sensitivity (LOD = 22.6 ng mL^{-1}), selectivity, and a linear range of 100–1000 ng mL^{-1}, and it was successfully applied to ophthalmic preparations and artificial aqueous humor samples, yielding high recoveries. This method provides a cost-effective, sensitive alternative to chromatographic and electrochemical methods for Bfln.HCl determination, eliminating the need for complex derivatization, expensive reagents, or nanomaterials fabrications. Green analytical chemistry assessments (AGREE, BAGI, and RGB models) confirmed the method's environmental sustainability. This study offers both experimental and computational insights into the fluorescence enhancement mechanism of Bfln.HCl, laying the groundwork for extending this approach to other fluoroquinolone antibiotics.

1. Introduction

Fluoroquinolones, a class of organic halogen compounds, have attracted considerable attention for their broad-spectrum antimicrobial and chemotherapeutic applications [1–5]. Besifloxacin hydrochloride (Bfln.HCl), a topical fluoroquinolone antibiotic, is chemically designated as 7-[(3R)-3-aminohexahydro-1H-azepin-1-yl]-8-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Fig. 1), forming a hydrochloride salt at its primary amine group [6]. Bfln·HCl is primarily used to treat bacterial conjunctivitis caused by gram-positive and gram-negative pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* [7–9]. Its ocular suspension formulation enhances targeted delivery, minimizes systemic exposure, and reduces the risk of antimicrobial resistance [10]. Bfln·HCl exerts its antibacterial effect by inhibiting DNA gyrase and topoisomerase IV, enzymes critical for bacterial DNA replication and repair [11,12]. In comparison to earlier fluoroquinolones, its dual-target method improves efficacy while reducing resistance development [13]. It is generally well tolerated, with adverse effects typically limited to localized irritation [7].

Several analytical methods have been developed for Bfln.HCl determination, including chromatographic techniques [5,14–20], electrochemical method using gold nanoparticle-modified electrodes [21], spectrophotometry [22,23], and spectrofluorimetry [24,25]. However, chromatographic approaches are often costly, complex, and time-consuming. Although electrochemical methods are generally considered simple and low-cost, the reported electrochemical method for determining Bfln.HCl needs complex derivatization steps, expensive reagents, and nanomaterial modifications, potentially limiting their routine application. Moreover, prior spectrofluorimetric methods required expensive derivatization agents or suffered from limited selectivity due to quenching mechanisms. Therefore, a simpler, cost-effective, and highly sensitive alternative remains necessary.

Fluorescence-based determination methods offer notable advantages, including high sensitivity, cost-effectiveness, and simplicity compared to chromatographic and electrochemical methods [26–28]. Moreover, they eliminate the need for complex derivatization steps, expensive reagents, and nanomaterial modifications required in previous electrochemical methods. Many fluoroquinolones, such as gatifloxacin [29], norfloxacin [30], gemifloxacin [31], and others [32–34], exhibit enhanced intrinsic fluorescence in acidic media. However, none of these reported studies explained the exact mechanism for this fluorescence enhancement. Notably, no previous study has systematically investigated the fluorescence behavior of Bfln.HCl or provided theoretical insights into its enhancement in acetic acid medium.

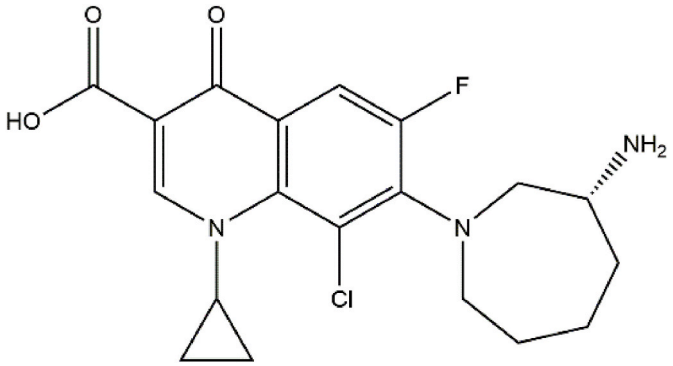


Fig. 1. Chemical structure of besifloxacin.

Herein, we report a novel spectrofluorimetric method for Bfln.HCl determination based on intrinsic fluorescence enhancement in acetate buffer, supported by computational chemistry. Density functional theory (DFT) calculations reveal that fluorescence enhancement arises from the formation of stable 1:2 Bfln+/AcOH cyclic complexes. This work not only provides a sensitive, selective, and cost-effective method for Bfln.HCl quantification in ophthalmic formulations and artificial aqueous humor but also offers the first theoretical insight into its fluorescence behavior. It offers a practical and efficient tool for pharmaceutical quality control and clinical analysis. Furthermore, the method's environmental impact was assessed using Analytical GREEnness (AGREE), Blue Applicability Grade Index (BAGI), and Red-Green-Blue (RGB) evaluation tools, confirming its greenness, blueness, and overall whiteness.

2. Experimental

2.1. Chemicals and instrumentation

Bfln.HCl (purity 99.50 %) was acquired from EVA Pharma for Pharmaceuticals (Haram, Gaza, Egypt). Ocubesiflox® eye drop (6.0 mg/mL) was purchased from a pharmacy in Fayoum City, Egypt. Ethyl alcohol, methyl alcohol, acetone, acetonitrile, acetic acid, sodium hydroxide and hydrochloric acid, were obtained from (El-Nasr Chemical Co., Cairo, Egypt).

Fluorescence measurements were carried out using a Jasco® FP-750 spectrofluorometer (Tokyo, Japan). The slit widths of both the excitation and emission monochromators were set to 10 nm. The excitation wavelength was fixed at 297 nm, and the emission spectra were recorded over the range of 350–500 nm. Centrifuge 5000 rpm (HERMLE, model Z206 A, Germany) was used.

2.2. Bfln.HCl stock solution preparation

Ten milligrams of standard Bfln.HCl powder was weighed and dissolved in 100 mL of distilled water. This solution was then further diluted with distilled water to obtain a series of working solutions with a concentration range of 1.0–10.0 μg mL^{-1}. The standard and working solutions were stable for at least one week when kept in the refrigerator.

2.3. Bfln.HCl determination

2.3.1. Assay of Bfln.HCl in standard form

In this procedure, 1.0 mL from the working solution (1.0–10.0 μg mL^{-1}) was transferred into 10-mL calibrated test tubes and then 2.5 mL of acetate buffer pH 4.0 was added. Subsequently, the tubes were completed to the marks with distilled water; hence, the final concentrations of Bfln.HCl was in the range of 100–1000 ng mL^{-1}. Finally, the fluorescence response of the final solution was obtained at 440 nm, following excitation at 297 nm. Additionally, a parallel blank experiment was performed, involving the same procedural steps but excluding the addition of the Bfln.HCl drug.

2.3.2. Assay of Bfln.HCl in eye drop sample

To assay Bfln.HCl in eye drops, a volume equivalent to 1.7 mL of Ocubesiflox® eye drops was transferred into a 100 mL volumetric flask along with 60 mL of distilled water and mixed thoroughly. This solution was then adjusted to a final volume of 100 mL using distilled water and then it was filtrated several times to obtain a clear solution. The clear solution was further diluted with the same solvent to achieve Bfln.HCl concentrations that are suitable for the method's range. Precise volumes of the resulting solution were assessed by following the fluorescence assay procedures as mentioned above under section 2.4.1.

2.3.3. Assay of Bfln.HCl in spiked artificial aqueous humor

Artificial aqueous humor was prepared according to the previous study by Fekry and coworkers [35]. The aqueous humor test solution was prepared by dissolving specific electrolytes, glucose, urea, albumin, and other components in water. The ingredients were added sequentially, and the pH was adjusted to 7.21 using 1.0 M HCl. After filtration, the solution was stored at −20 °C until further analysis. The prepared aqueous humor was diluted 100-fold with distilled water to eliminate matrix interference. Various volumes of Bfln.HCl standard solution (equal to these concentrations; 1.0, 1.5 and 2.0 μg mL^{-1}) were added to 1.0 mL of artificial aqueous humor solutions and mixed thoroughly. Subsequently, 2.5 mL of acetate buffer pH 4.0 was introduced and thoroughly mixed. After that, the total volume was adjusted to 10.0 mL with distilled water. Then, the fluorescence response was obtained at 440 nm. Concurrently, a control experiment was performed using aqueous humor without any drug under the same conditions.

3. Results and discussion

3.1. Fluorescence behavior of Bfln.HCl in acetate buffer

Fig. 2A shows the absorption spectra of Bfln.HCl before and after the addition of acetate buffer (pH = 4.0). It is clearly observable that there is a redshift equal to 10 nm in the λ_{max} of the Bfln.HCl after the addition of acetate buffer (pH = 4.0). Also, the fluorescence behavior of Bfln.HCl was investigated. Fig. 2B represents the fluorescence response of Bfln.HCl before and after the addition of acetate buffer (pH = 4.0). Upon the addition of acetate buffer (pH = 4.0), the fluorescence intensity of Bfln.HCl at 440 nm significantly increased. This phenomenon was further investigated using other fluoroquinolones (e.g., gatifloxacin, gemifloxacin, moxifloxacin, and nadifloxacin) under identical experimental conditions. The results (Table S1) demonstrate that acetate buffer markedly enhances the fluorescence intensity of these compounds.

The observed fluorescence enhancement is likely due to the formation of stable cyclic complexes between Bfln.HCl and AcOH. These interactions primarily occur at C2–H/C3–CO and C4═O/C5–H of the fluoroquinolone scaffold, leading to enhanced electronic delocalization and an observable redshift in λ_{max}. Later, we will clearly clarify this fluorescence-enhancement mechanism by computational investigations of the frontier orbitals of Bfln.HCl along with its complexes with acetic acid at different structural sites and different molar ratios.

3.2. DFT investigation of the origin of enhanced fluorescence of Bfln upon association with acetic acid

Considering the previously reported conformational diversity of alicyclic compounds of medicinal and health interest [3,36–39], an accurate DFT model was employed on the studied structures. The model utilized the B3LYP-D3 hybrid functional [40] with restricted spin Becke-Johnson damping dispersion corrections [41] and a split-valence triple-ζ basis set 6-311+G(d,p)), following the reported method [42]. Additionally, the Conductor-like Polarizable Continuum Model (CPCM) solvation [43] was applied to simulate aqueous solvation, ensuring alignment with the real fluorometric experiment conditions.

After successfully optimizing Bfln+ (cationic form of the Bfln.HCl) structure as seen in Scheme 1, the interaction with acetic acid was studied at different molar ratios. The DFT investigation results revealed three points available for the coordination of acetic acid molecules within the structure of Bfln+. Considering the dual association capacity of carboxylic acid functionality of Bfln+ (C═O oxygen is H-bond acceptor and OH is H-bond donor), a cyclic complex (8-center cyclic complex) was likely to form at three structural points in Bfln + structure

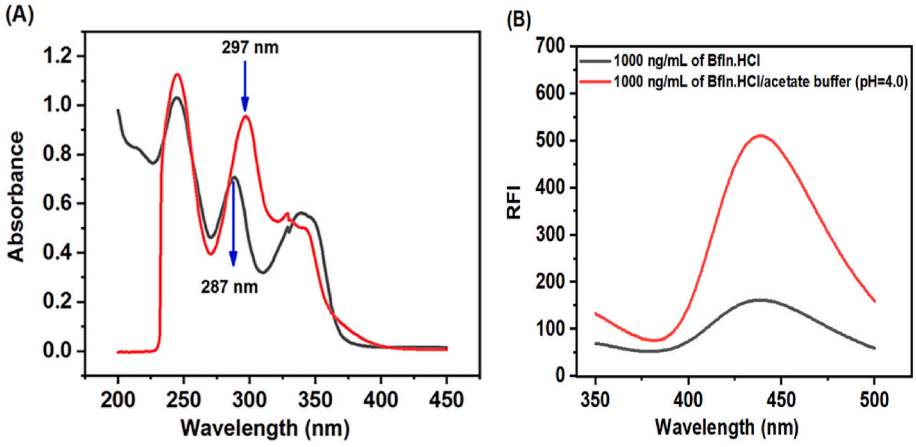
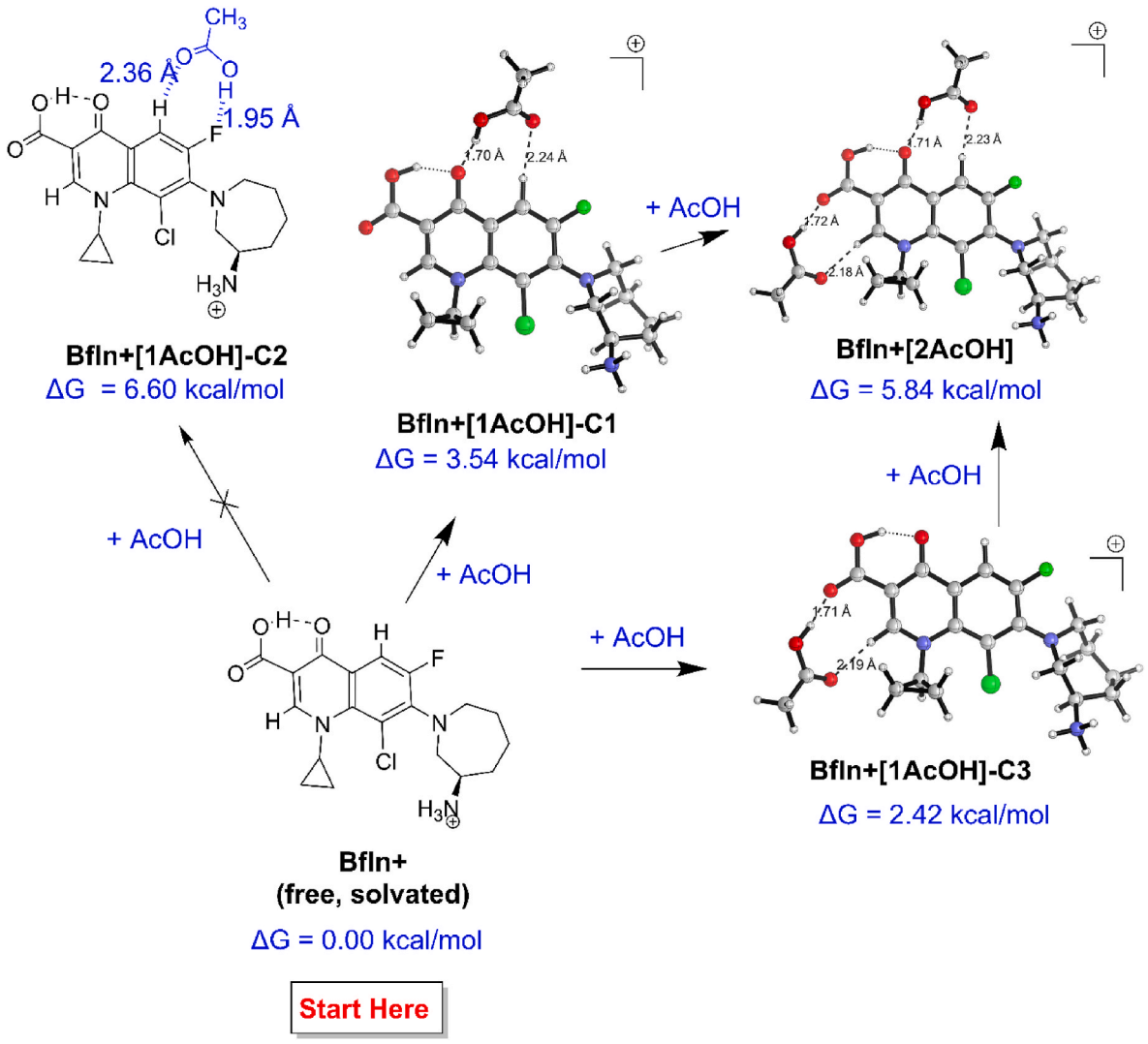


Fig. 2. (A) Absorption spectrum of 20 μg mL^{-1} Bfln.HCl before (black color) and after (red color) the addition of acetate buffer (pH = 4.0). (B) Fluorescence spectra of 100 ng mL^{-1} of Bfln.HCl in the presence and absence of acetate buffer of pH 4.0 at (λ_{em} = 440 nm) and (λ_{ex} = 297 nm). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



Scheme 1. Schematic Diagram shows the three possibilities of 1:1 host/guest coordination between Bfln+ and acetic acid (AcOH). Stable 8-center cyclic complexes of the 1:1 type are possible with **Bfln + [1AcOH]-C3** being the most likely. Further association at a 1:2 host/guest molar ratio is also possible (**Bfln + [2AcOH]**). ΔG represents the relative Gibbs Free Energies (G) as compared to the reference **Bfln.AcOH** structure (with ΔG = 0.00 kcal/mol).

(C3–CO, C4═O and C6–F, Scheme 1). Accordingly, a thermodynamic study of the three possibilities of 1:1 Bfln+/AcOH coordination complex formation was carried out. Interestingly, the results revealed the following complexes' stabilities in descending order: **Bfln + [1AcOH]-C3** > **Bfln + [1AcOH]-C1** > **Bfln + [1AcOH]-C2** (corresponding to C3–CO, C4═O and C6–F respectively). The exact H-bond lengths of the three complexes are depicted in Scheme 1.

Surprisingly, fluorine coordination seems to be less probable (**Bfln + [1AcOH]-C2**), and this could be attributed to the more favored complexes formed by sp2-hybridized oxygen (**Bfln + [1AcOH]-C1** and **Bfln + [1AcOH]-C3**) in which the cyclic H-bonded complex is assisted by resonance effect of the oxygen π-cloud. Looking at the thermodynamic barriers of formation of the three 1:1 complexes (**ΔG** = 2.42–6.60 kcal/mol, Scheme 1), we can safely assume that all of them would exist in a rapid equilibrium, where the free Bfln+ and AcOH molecules are slightly more predominant due to extensive aqueous solvation.

Notably, the formation of the three complexes between $Bfln^+$ and acetic acid is thermodynamically unfavored. This could be best justified by suggesting that water molecules individually solvate $Bfln^+$ and AcOH more efficiently than they could by $Bfln^+$/AcOH complex formation. This is due to the charged nature of Bfln+, the multiple polar functional groups, and the small size of the water molecule that allows for the formation of solvent shells. However, the reduced complexation efficiency of acetic acid compared to water is still low (2.4–6.6 kcal/mol, Scheme 1). It is worth noting that all the DFT calculations employed CPCM solvation using water as a solvent to simulate the aqueous solvation process, following a previous work [44].

At this point, we found it is logical to calculate the frontier molecular orbital energy gaps ($\Delta G_{[LUMO\text{-}HOMO]}$) of the three 1:1 Bfln+/AcOH complexes to be compared with the experimental values calculated from the following equations (1) and (2).

$$\Delta G_{[LUMO\text{-}HOMO]}\ (eV) = h \times c/\lambda_{exc} \ldots \ldots \ldots \ldots [45] \qquad \text{equation (1)}$$

$$\Delta G_{[LUMO\text{-}HOMO]}\ (a.u.) = \Delta G_{[LUMO\text{-}HOMO]}\ (eV) / 27.21 \ldots \ldots \ldots \ldots \ldots \text{equation (2)}$$

where $\Delta G_{[LUMO\text{-}HOMO]}$ is the energy gap between the frontier orbitals of a given molecule or complex (in eV and a.u., respectively in equation (1) and 2), h represents Planks Constant, *c* is the speed of light, λ_{exc} is the wavelength of excitation and h × *c* is a constant equal to 1239.84193 eV nm. a.u. is the atomic unit.

Upon applying the above-mentioned equations to the experimental wavelengths of excitation (λ_{exc}) of Bfln.HCl salt solution before and after subjecting it to the optimum fluorescence enhancement conditions (Fig. 2A), we could successfully obtain the following experimental

results. The values of λ_{exc} [Bfln+] = 287 nm and λ_{exc} [Bfln+/AcOH] = 297 nm correspond to $\Delta G_{[LUMO\text{-}HOMO]}$ [Bfln+] = 0.158765407 a.u. and $\Delta G_{[LUMO\text{-}HOMO]}$ [Bfln+/AcOH] = 0.1534197704 a.u., respectively. In other words, a decrease in the energy gap of the frontier orbitals of the Bfln + would be expected to occur after complexation with acetic acid at a value of $\Delta\Delta G_{[LUMO\text{-}HOMO]}$ [complexed/free] = −0.00535 a.u. (Fig. 3).

Hence, a fast comparison of the computed $\Delta G_{[LUMO\text{-}HOMO]}$ values of both the 1:1 Bfln+/AcOH complexes and the free Bfln + forms afforded a value of $\Delta\Delta G_{[LUMO\text{-}HOMO]}$ [1:1 complexed/free] = −0.00407 a.u. for **Bfln + [1AcOH]-C1** which is still 24 % far from reality. However, there is a difference between calculated and computed energy, but the 1:1 complex **Bfln + [1AcOH]-C1** seems to account for most of the experimentally observed enhancement of fluorescence activity of Bfln.HCl salt solution upon treatment with the acetate buffer.

Moreover, we have studied the possibility of 1:2 Bfln+/AcOH complexation (**Bfln + [2AcOH]**). As shown in Fig. 3, the computed $\Delta\Delta G_{[LUMO\text{-}HOMO]}$ [1:2 complexed/free] in this case was found to come closer to the real value (−0.00483 vs. −0.00535 a.u.). Notably, the 1:2 complex (Bfln + [2AcOH]) exhibits the lowest HOMO-LUMO energy gap among all tested structures ((**Bfln + [1AcOH]-C3**, **Bfln + [1AcOH]-C1** and **Bfln + [1AcOH]-C2**)), closely matching experimental values. This suggests that the dual AcOH complexation enhances fluorescence intensity. However, additional complexation with AcOH molecules (1:3 or 1:4 complexes) leads to overcrowding, disrupting the electronic interactions responsible for fluorescence enhancement. This close agreement between calculated and computed energy gaps confirms that **Bfln + [2AcOH]** is the primary species responsible for fluorescence enhancement. The lower energy gap in the 1:2 complex suggests that dual complexation enhances charge delocalization, leading to higher fluorescence efficiency via host/guest chemistry rather than the usual acid/base chemistry.

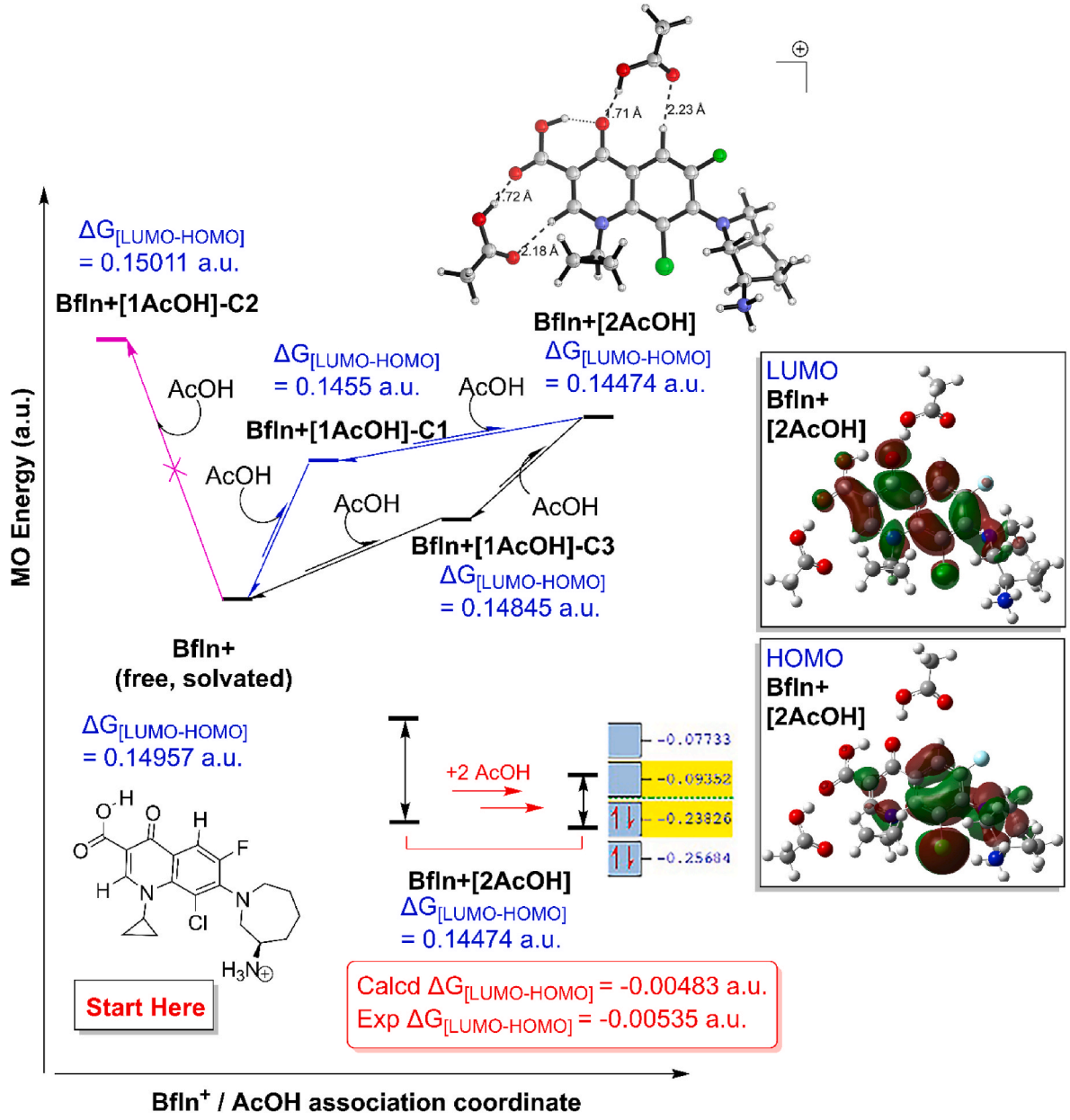


Fig. 3. Frontier orbital diagram showing the values of LUMO/HOMO energy gaps at each stage along the **Bfln** + /AcOH complexation coordinate. A comparison with the experimental data leads to a conclusion that the decrease in the frontier orbital energy gap ($\Delta\Delta G_{[LUMO\text{-}HOMO]}$) is most expected to be attributed to **Bfln** + /AcOH complexation of 1:2 type (**Bfln + [2AcOH]**) giving values very close to the experimental ones.

3.3. Optimizing fluorescence measurements for Bfln.HCl

To fine-optimize the fluorescence measurement of Bfln.HCl, we systematically assessed diverse factors influencing emission intensity. First, we examined the effect of pH using Britton-Robinson buffer to evaluate its impact on the drug's fluorescence response. Interestingly, a slightly acidic pH (3.8–4.5) produced a significantly stronger fluorescence signal compared to higher pH values (Fig. S1A). Next, we compared the fluorescence intensities of Bfln.HCl in different buffer systems, including acetate buffer, Britton-Robinson buffer, and Teorell-Stenhagen buffer at a pH of 4.0. Among these, the acetate buffer yielded the highest fluorescence response and was therefore selected for subsequent measurements (Fig. S2). The superior fluorescence response in acetate buffer (pH 4.0) may be attributed to the increased stability of Bfln+/AcOH complexes. Then, we optimized the volume of acetate buffer (pH 4.0) and found that 2.5 mL provided the most enhanced fluorescence signal (Fig. S1B). Finally, we investigated the influence of different solvents, for instance, methanol, acetonitrile, water, acetone, and ethanol on fluorescence intensity. Notably, distilled water was identified as the optimal solvent, providing the maximum signal intensity (Fig. S1C). In conclusion, by optimizing pH, selecting acetate buffer, and using distilled water as the solvent, we established a highly sensitive spectrofluorimetric method for the accurate determination of Bfln.HCl.

3.4. Method validation

3.4.1. Calibration curve

We have applied this fluorimetric approach for determining Bfln.HCl in its standard form. Several concentrations of Bfln.HCl solutions as final concentrations (100–1000 ng mL^{-1} for standard solutions were prepared for constructing the calibration curve. Table 1 lists different analytical performance parameters for the determination of Bfln.HCl in its standard solutions.

3.4.2. Sensitivity, detection limit, and quantification limit

The sensitivity of an analytical method refers to its ability to detect small changes in the analyte concentration relative to background noise. It is typically quantified as the slope of the calibration curve, which represents the change in the analytical signal per unit change in analyte concentration. The limits of detection (LOD) and quantification (LOQ), which are indicative of the sensitivity of the method, were calculated based on the standard deviation of the intercept and the slope of the linear calibration curve. The LOD was expressed as 3.3 σ/S, while LOQ was expressed as 10 σ/S, where (σ) is the standard deviation of the intercept and (S) is the sensitivity parameter expressed by the slope of the calibration curve. LOD and LOQ for Bfln.HCl were calculated to be 22.60 and 68.50 ng mL^{-1}, respectively. Notably, our method revealed high sensitivity and low detection limit when compared with the previously reported methods (Table 2).

Table 1
Analytical performance data for Bfln.HCl analyzed by the proposed spectrofluorimetric method.

Parameter	Data for Bfln.HCl
λ_{ex} (nm)	297
λ_{em} (nm)	440
Concentration range (**ng mL^{-1}**)	100–1000
LOD (**ng mL^{-1}**)	22.60
LOQ (**ng mL^{-1}**)	68.50
Determination coefficients ($\mathbf{R^2}$)	0.9996
Correlation coefficient (r)	0.9997
Slope (b)	0.731
Intercept (a)	6.14
Standard deviation of intercept (s_a)	4.96
Standard deviation of slope (s_b)	8.0×10^{-3}

3.4.3. Precision and accuracy

To evaluate the accuracy of the proposed fluorimetric method, the standard addition technique was followed. The obtained high recovery values (98.73 ± 0.90 % to 100.30 ± 1.60 %), as presented in Table 3, confirm the high accuracy of the developed method. Additionally, the simplicity of the standard addition approach enhances the method's reliability in accurately quantifying the studied drug.

To study the precision of the developed method, six determinations at three concentration levels 100, 500, and 1000 ng mL^{-1} were utilized. Intra-day precision was evaluated by performing replicate measurements of the samples at different time intervals on the same day, while Inter-day precision was assessed by analyzing the same concentration ranges through three successive days. Intra-day and inter-day precision were revealed in terms of RSD as recommended by the ICH guidelines. As shown in Table 4, all tested concentrations displayed relative standard deviations (%RSD) below 2.0 %, signifying the high precision of the developed method.

3.4.4. Selectivity study

The method's selectivity was assessed by investigating potential interference from excipients commonly added in pharmaceutical ophthalmic formulations and in the presence of different interfering compounds naturally present in the aqueous humor. For this evaluation, samples containing a known quantity of Bfln.HCl (6.0 mg) mixed with benzalkonium chloride in a 50:50 ratio were prepared and dissolved in water. The method was then applied to analyze these laboratory-prepared samples using the standard procedure. The recovery value was found to be 99.20 ± 1.30 % demonstrating the absence of interference from the excipient, highlighting the method selectivity. Additionally, the method's selectivity was verified by analyzing aqueous humor samples to identify any potential interference. The interfering species investigated in this study were glucose, urea, ascorbate, different amino acids, K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^- and HCO_3^-. As represented in Table S2, the effect of the interfering species is negligible, confirming the selectivity of the presented approach for monitoring Bfln.HCl in real samples. Furthermore, analysis of Bfln·HCl in the presence of other quinolones demonstrated high selectivity, attributed to the distinct emission wavelengths of Bfln·HCl compared to the other tested quinolones (Table S1). In addition, the effect of albumin on the fluorescence intensity of Bfln·HCl was evaluated. The results indicated that albumin induces fluorescence quenching of Bfln·HCl, consistent with previous reports on the interaction between Bfln·HCl and albumin [46].

3.5. Analysis of Bfln.HCl in ophthalmic drops

The validated fluorimetric method was effectively employed for the assessment of Bfln.HCl in eye drop formulation, representing its practical applicability. Table 5 shows the recovery results for Bfln.HCl analysis, comparing the newly developed method with a previously published approach [18]. As shown in Table 5, the outcomes from both methods reveal no significant differences, indicating that the accuracy and precision of the developed method are comparable to those of the referenced technique.

3.6. Monitoring of Bfln.HCl in spiked artificial aqueous humor

The highly sensitive method was employed to quantify Bfln.HCl in spiked artificial aqueous humor. The aqueous humor samples were spiked with different concentrations of Bfln.HCl, and then the developed method was applied. The evaluation of emission responses consistently demonstrated acceptable recoveries (100.34 ± 1.30 % to 99.60 ± 0.90 %). The significantly high recovery range, as shown in Table 6, indicates the effectiveness of this method in measuring Bfln.HCl levels in spiked aqueous humor samples.

Table 2
Comparisons between the performance of the developed method and other reported methods for the determination of Bfln.HCl.

Method	Range (ng/mL)	LOQ/LOD (ng/mL)	Application	Remarks	Ref.
UPLC	750 - 3750	500/100	- Dosage form	- Expensive instrumentation - Moderate sensitivity - Hazardous solvents	[15]
Chiral HPLC	1000–6000	1000/200	- Enantiomeric purity	- Expensive - Precolumn derivatization - Hazardous solvents - Low sensitivity	[17]
HPLC	20,000–80,000	N/A	- Ophthalmic suspension	- Low sensitivity - Hazardous solvents	[18]
LC-MS/MS	2.0–2000	N/A	- Human tears	- Very expensive - Expert personnel - High sensitivity	[19]
HPLC-FD	5.0–1000	N/A	- Human tears	- Expensive instrumentation - Hazardous solvents	[20]
CV and DPV	866.40–21660	492.3/149.6	- Eye drops - Biological fluids	- Expensive electrode -Tedious preparation of working electrode	[21]
Spectrophotometry	3000–30,000	2600/880	- Simulated tears	- Low sensitivity	[22]
Spectrophotometry	2500 - 80,000	N/A	- Eye drops	- Low sensitivity	[23]
Fluorometry	200–1000	28.2/8.5	- Eye drops - Simulated tears	- Required derivatization - expensive reagent	[24]
Fluorometry	100–1000	22.6/68.50	- Ophthalmic formulations - Artificial aqueous humor	- Simple - No derivatization - Sensitive - Eco-friendly	Current work

CV, cyclic voltammetry; DPV, differential pulse voltammetry; HPLC-FD; high-performance liquid chromatography-fluorescence detection; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; UPLC, ultra-performance liquid chromatography.

Table 3
Accuracy study by evaluation of recoveries of the spiked eye drop samples.

Dosage form	Concentration taken (ng/mL)	Concentration added (ng/mL)	Total concentration found (ng/mL)	% Recovery[a] ± SD
Ocubesiflox® Eye drops	100.0	0	100.30	100.30 ± 1.60
		50.0	148.10	98.73 ± 0.90
		100.0	198.50	99.25 ± 1.20
		150.0	250.69	100.27 ± 0.95

[a] Mean of three determinations.

Table 4
Precision results for Bfln.HCl in standard form analyzed by the proposed spectrofluorimetric method.

Parameter	Bfln.HCl	
Conc. (ng mL^{-1})	**% Recovery[a] ± SD**	**RSD**
Intra-day precision		
100.0	99.20 ± 1.85	1.86
500.0	99.50 ± 0.63	0.63
1000.0	100.90 ± 1.30	1.28
Inter-day precision		
100.0	98.30 ± 1.20	1.22
500.0	98.55 ± 0.95	0.96
1000.0	99.50 ± 0.50	0.50

[a] Mean of three determinations.

Table 5
Determination of Bfln.HCl in eye drops using the proposed and reported methods.

Dosage form	Labeled content	% Recovery[a] ± SD		t-value[b]	F-value[b]
		Proposed method	Comparison method [18]		
Ocubesiflox® Eye drop	**(0.6 %)**	99.35 ± 1.43	99.54 ± 1.15	0.22	1.57

[a] Mean of three determinations.
[b] Theoretical values at 95 % confidence limit: $t = 2.31$, $F = 6.39$.

Table 6
Determination of Bfln.HCl in spiked artificial aqueous humor by the proposed method.

Added amount (**ng** mL^{-1})	Found amount (**ng** mL^{-1})	% Recovery[a] ± SD
100.0	100.34	100.34 ± 1.30
150.0	149.40	99.60 ± 0.90
200.0	200.20	100.10 ± 1.20

[a] Mean of three determinations.

3.7. Greenness, blueness, and whiteness assessment of the developed method

The greenness, blueness, and whiteness evaluations of the proposed method were implemented to check the eco-friendliness, analytical performance, and practical effectiveness of the proposed spectrofluorimetric method. For the greenness evaluation, Analytical GREEnness (AGREE) was employed to estimate the greenness of our developed method. AGREE [47] is a comprehensive, simple, and efficient metric approach that provides easily interpretable and meaningful outcomes. It has been widely utilized to estimate the greenness of various analytical methodologies with supreme efficiency [48,49]. In brief, the AGREE program can determine the color and a scale in the 0–1 range for each principle of the twelve principles, which depicts its degree of greenness. Fig. 4A illustrates the clock-like AGREE graph, with a total score of 0.83 which indicates the greenness of the developed method.

For the blueness evaluation, the metric tool Blue Applicability Grade

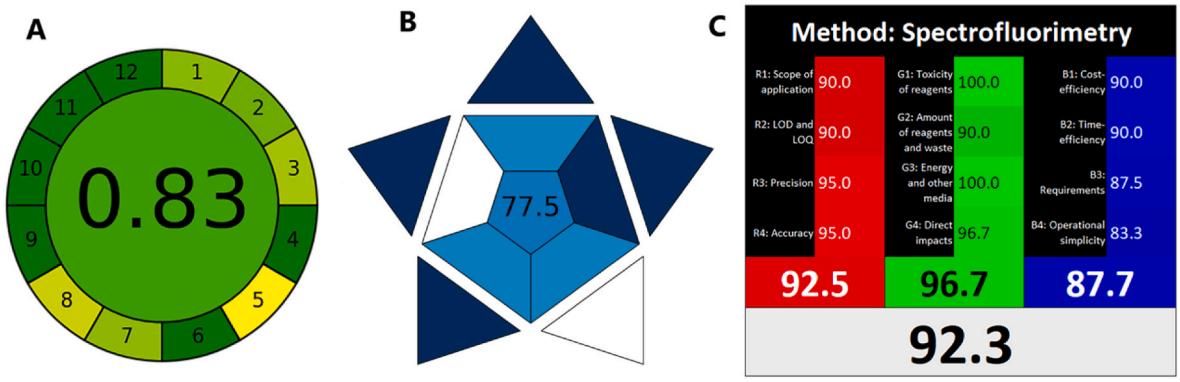


Fig. 4. Greenness, blueness, and whiteness evaluation of the developed method using (A) AGREE metric, (B) BAGI metric, and (C) RGB model, respectively.

Index (BAGI) was used to estimate the applicability of the developed method. This tool provides a quantitative assessment of the suitability and functionality of the analytical technique to be employed for practical applications [50]. The range of the BAGI score lies between 25 and 100 where the high score (near 100) indicates high applicability of the analytical method employed in a specific study. Our developed fluorescent method indicated a high score of BAGI (Fig. 4B), which indicates high blueness (applicability).

For the whiteness evaluation, the RGB comprehensive model was used to demonstrate how the analytical performance (represented in red color), safety and eco-friendliness (represented in green color), and practicality (represented in blue color) of the developed method were covered [51,52]. Fig. 4C demonstrates the whiteness of our proposed method since it produced a 92.5 % score for analytical performance (red), 96.7 % score for eco-friendliness (green), 87.7 % score for practicality (blue), and an overall score of 92.3 % (white).

4. Conclusion

In this study, we developed a highly sensitive and selective spectrofluorimetric method for the quantification of Bfln.HCl, leveraging intrinsic fluorescence enhancement in acetate buffer. The optimized conditions, including pH adjustment, buffer selection, and solvent choice, significantly improved the fluorescence intensity, enabling accurate and reproducible determination. The proposed method's linear range was 100–1000 ng mL^{-1}. To elucidate the fluorescence enhancement mechanism, DFT calculations were employed. The theoretical findings revealed that Bfln + forms stable 1:2 cyclic complexes with AcOH, leading to HOMO-LUMO energy gap reduction, which directly correlates with the observed fluorescence enhancement. The close agreement between computed and experimental values supports this complexation-driven mechanism. Furthermore, the green analytical chemistry assessment (AGREE, BAGI, RGB models) confirmed the method's environmental sustainability, making it a viable alternative to chromatographic and electrochemical techniques that require expensive reagents and lengthy procedures. Overall, this study provides a cost-effective, highly sensitive, and eco-friendly approach for Bfln.HCl quantification, with potential applications in pharmaceutical quality control and clinical analysis. Additionally, the findings offer fundamental insights into fluorescence enhancement mechanisms, which could be extended to other fluoroquinolone antibiotics, paving the way for future advancements in spectrofluorimetric drug analysis.

CRediT authorship contribution statement

Abobakr A. Mohamed: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Raed M. Maklad:** Writing – review & editing, Software, Methodology, Data curation, Conceptualization. **Abdallah M. Zeid:** Writing – review & editing, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Islam M. Mostafa:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2025.344446.

Data availability

Data will be made available on request.

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