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ORIGINAL PAPER

Resolution of Ofloxacin–Ciprofloxacin and Ofloxacin–Norfloxacin Binary Mixtures by Flow-Injection Chemiluminescence in Combination with Partial Least Squares Multivariate Calibration

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Abstract A flow-injection chemiluminescence (CL) method is described for the determination of ciprofloxacin (CIP), norfloxacin (NOR) and ofloxacin (OFL), commonly used antibiotics of the fluoroquinolones family. The method is based on the CL reaction of the fluoroquinolones with tris (2,2′-bipyridyl) ruthenium(II) and Ce (IV), in sulfuric acid medium. The maximum CL emission, given at 0.45 min for CIP, at 0.35 min for NOR and at 0.04 min for OFL, respectively, were measured, allowing the simple application of the proposed method to the routine analysis of the antibiotics. The methods were applied to the determination of CIP, NOR and OFL, in several pharmaceutical preparations, with very satisfactory results, and validated by a previously reported HPLC method. The time-resolved equipment allowed the measurement of the kinetic evolution of the chemiluminescence signals. In base to the differences in the kinetic behaviour of ofloxacin with respect to ciprofloxacin and norfloxacin, binary mixtures of the drugs were resolved by using the time-resolved chemiluminescence signals, in combination with first-order partial least-squares (PLS) multivariate calibration.

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Introduction

Fluoroquinolones are important antibacterial agents developed in the 1980s, and have many applications in veterinary and human medicine. The pharmaceuticals have a broad spectrum of activity and good oral absorption [1]. The great advantage of these drugs, in order of activity and spectral characteristics, is due to the presence of a fluorine atom in position six of the quinolonic ring. The introduction of the fluorinated quinolones represents important therapeutic advantages, because this group of antibiotics shows higher antibacterial activity than the parent compounds [2]. There is concern about the possibility of exposure to low levels of these compounds resulting in the development of resistance of human pathogens to antibiotics [3].

Ciprofloxacin (CIP) [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(piperazinyl)-quinolone-3-carboxylic acid], Fig. 1, belong to the fluoroquinolones family, which are bacteriostatic at low concentration and bactericidal at high concentrations. CIP is approved for use in the treatment of bone and joint infections, infectious diarrhea caused by *Shigella* or *Campylobacter*, lower respiratory tract infections, skin infections, and urinary tract infections. In addition, it has found off-label use as an alternative drug for the treatment of gonorrhea, salmonella, and yersinia infections [4, 5]. In general, CIP is active against

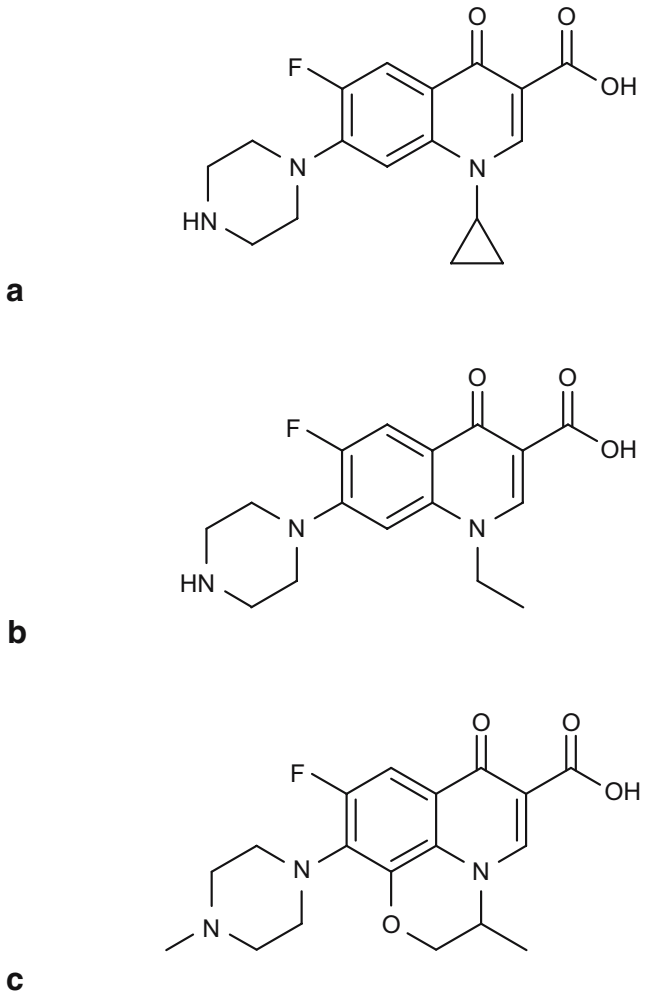

Fig. 1 Structure of **a** ciprofloxacin, **b** norfloxacin, **c** ofloxacin

susceptible gram-negative and gram-positive aerobic bacteria, so therefore it should not be used alone for mixed aerobic–anaerobic bacterial infections [5, 6].

Norfloxacin (NOR) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7(piperazin-1-yl)quinoline-3-carboxylic acid], Fig. 1, is used in a wide range of gastrointestinal, urinary and respiratory tract infections; ocular and skin infections as well as in patients with intraabdominal infections in combination with anti-anaerobic agents [7–9].

Ofloxacin (OFL) (±)9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-pieraziny)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid, Fig. 1, is a synthetic fluorinated quinolone derivative having activity against both gram negative and gram positive bacteria through inhibition of their DNA gyres [10]. It is widely used in the treatment of respiratory tract and urinary tract infections [11].

There is much interest in determining fluoroquinolones for the purpose of pharmaceutical quality control and numerous techniques have been developed. Most of the analytical methods for the determination of fluoroquinolones employ HPLC. In 1998, Carlucci reported a review of the published HPLC assays that used UV or fluorescence detection [12]. Later on, other works have been reported employing UV detection [13, 14] and fluorescence detection [15–20]. Other reported methods include: spectrophotometry [21–24], fluorimetry ([25], capillary electrophoresis [26–29], and immunoassay [30].

Chemiluminescence (CL), an analytical useful chemical property, has aroused much interest for spectroscopic detection on account of its inherent sensitivity and selectivity. Liquid-phase chemiluminescence (CL) reactions have been used to determine a wide range of analytes from trace metals to pharmaceuticals. Analytically, these reactions are attractive on the grounds of the excellent limits of detection potentially derived from the absence of source noise and scatter, the high selectivity resulting from the limited number of available CL reactions and the wide linear ranges that can be obtained. These CL reactions have been used for the sensitive and selective detection in a wide range of analytical techniques including flow injection analysis (FIA), sequential injection analysis (SIA), high performance liquid chromatography (HPLC) and capillary electrophoresis. Various sample-reagent mixing modes have been used in combination with a detector to record the chemiluminescence signal. Thus, a stopped-flow mixing module coupled to a CL detector, was used in conjunction with formation and decay rates of the CL profiles as kinetic measurement parameters [31, 32].

Regarding the determination of fluoroquinolones by flow-injection chemiluminescence, the cerium-sulfite, alone and sensitized by Tb^{3+}, potassium permanganate-sulfite sensitized by Tb^{3+}, potassium permanganate-thiosulfate, peroxynitrous acid, luminol-H_2O_2, tris-(1,10-phenanthroline) ruthenium(II)-cerium (IV) and tris(2,2′-bipyridyl) ruthenium (II)-cerium (IV) system, have been used.

The cerium-sulfite system has been proposed for the determination of CIP [33, 34], OFL [34, 35] and NOR [34] in pharmaceutical preparations. This later reaction served as the base of a molecular imprinting polymer system for NOR determination in urine samples [36]. The same system, sensitized by Tb^{3+}, allowed lowering the detection limits of the proposed methods for CIP [37] and NOR [38] in pharmaceutical preparations, extending the procedure to urine and serum samples.

The potassium permanganate-sodium sulfite, sensitized by Tb^{3+}, was also employed for OFL [39] in pharmaceutical preparations and urine, and the potassium permanganate-sodium thiosulfate, for CIP determination in pharmaceutical preparations, serum and urine [40].

The peroxynitrous acid system, obtained by acidified H_2O_2 plus nitrite, was proposed for the determination of CIP, NOR and OFL [41] in pharmaceutical preparations and the luminol-H_2O_2 was employed for OFL determination [42].

The tris-(1,10-phenanthroline)ruthenium(II)-organic acids-Ce (IV) was proposed for CIP [43] and the tris(2,2′-bipyridyl) ruthenium(II) plus cerium (IV) for CIP, OFL and NOR [44] in pharmaceutical preparations, urine and plasma, and as an HPLC detection system for OFL determination in chicken tissues [45].

Since the initial discovery of this latter chemiluminescent system [46], its utility has only been exploited for a relatively number of analytical applications. Common of these applications is the production of the reactive oxidant, $Ru(bipy)_3^{2+}$, followed by reduction, by an analyte species, to produce and emission of light [47].

Time-resolved chemiluminescence, based on stopped-flow chemistry, ensure rapid, efficient mixing of chemiluminescent reagent and sample, immediately before reaching the detector, and an assembly for this novel tool was described in a previous paper [48]. In this paper, a CL method has been developed for the determination of CIP, NOR and OFL in commercial formulations. The method is based on the CL reaction of these drugs with $[Ru(bipy)_3^{2+}]$ and Ce (IV) in sulfuric acid medium. In addition, the chemiluminescence-time data pairs acquired with this system, allowed the use of the time-resolved chemiluminescence data in combination with multivariate calibration techniques, as partial-least-squares (PLS), for the resolution of binary mixtures of the fluoroquinolones. As far to our knowledge, this is the first time that stopped-flow time-resolved chemiluminescence data are explored for mixtures resolution by chemometric analysis, although a time-resolved chemiluminescence method for the simultaneous determination of pyruvic and tartaric acids has been described, based on the chemiluminescent reaction of $[Ru(bipy)_3^{2+}]$ with Ce (IV). The resolution was possible in this latter case, without resorting to chemometric techniques, as the pyruvic acid system gives the highest chemiluminescence intensity at 2 s, whereas the tartaric acid system gives its most intense chemiluminescence emission at 64 s [49].

Experimental

Reagents

All experiments were performed with analytical-reagent grade chemicals and pure solvents. Ultra pure water was obtained from a Milli-Q system.

CIP was obtained from Fluka and NOR and OFL from Sigma. Stock standard solutions of 100.0 mg l^{-1} were prepared in 5.0×10^{-3} M sulfuric acid (Panreac). Working solutions of different concentrations were prepared by dilution of the stock solutions with 5.0×10^{-3} M sulfuric acid. Exposure to direct sunlight was avoided. A 2.5×10^{-2} M Ce (IV) solution was prepared by dilution of ammonium cerium (IV) sulfate 2-hydrate (Panreac) in 4.0 M sulfuric acid. A 2.0×10^{-3} M $[Ru(bipy)_3^{2+}]$ solution was prepared by dilution of tris (2,2′-bipyridyl) dichloro-ruthenium (II) hexahydrate (Aldrich) in ultra pure water.

Apparatus and software

The flow system comprised the conventional manifold depicted in Fig. 2. The reactants (fluoroquinolone sample solution, Ce (IV) and $[Ru(bipy)_3^{2+}]$) were pumped through the three-line manifold by a Gilson Minipuls-3 peristaltic pump, which was computer-controlled. The pump rate was 10.5 ml min^{-1} and the reagents circulated in poly(tetra-fluoroethylene) (PTFE) flow tubes (Tygon, 0.5 mm i.d., acid-resistant) for mixing in the detector cell. At an appropriate time, the flow was stopped abruptly and chemiluminescence–time data pairs were acquired using a

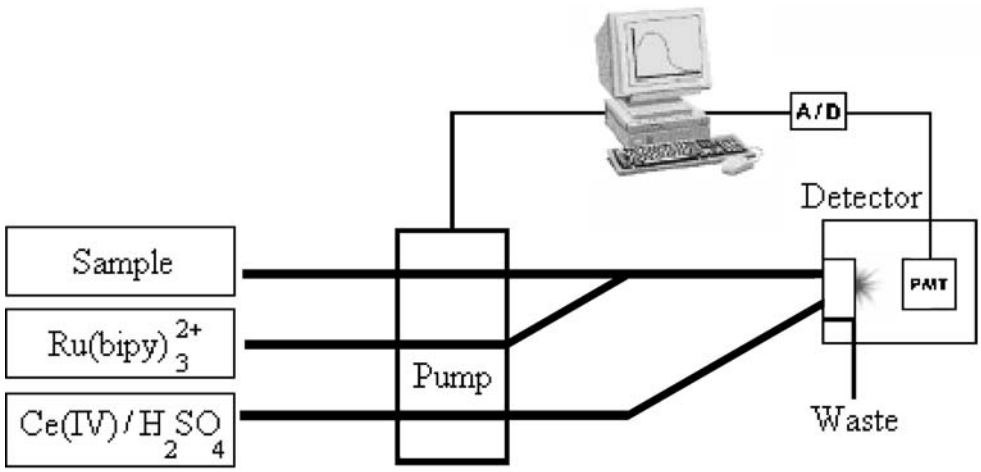

Fig. 2 Schematic diagram of the continuous-flow manifold

Camspec chemiluminescence detector CL–2 (photosensor module Hamamatsu 45773-20 spectral response from 300 to 600 nm; spiral–type flow cell, volume 120 μl; Sawston, Cambridge). The detector was interfaced to a computer via an analogue/digital converter; data were acquired using the chromatography station for Windows CSW32 software (Data Apex Ltd., Prague, Czech Republic) and processed to obtain parameters such as emission intensity at a fixed time and maximum emission intensity, by using a home-made software developed in our laboratory (J. A. Murillo Pulgarín, University of Castilla La Mancha, Spain).

Procedure

The continuous-flow manifold used to implement the stopped-flow system is described in Fig. 2. The sample stream was merged with another stream of 8.0×10^{-4} M [Ru $(bipy)_3^{2+}$] and the resulting stream was mixed with 1.75×10^{-3} M Ce (IV) (diluted in H_2SO_4 0.5 M). The merging point was a planar coiled quartz flow cell located in front of the photomultiplier tube (PMT) of a flow-through Camspec chemiluminescence detector CL-2. The flow-through system was stopped for three minutes. In this way, the reaction took place in the flow-cell. The system was interfaced to a computer via an A/D converter, and the CL transient signal was continuously monitored to obtain a plot of CL intensity versus time. All measurements were made at room temperature (18–20°C).

Data were acquired using the chromatography station for Windows CSW32 software. Each solution was assayed in triplicate and the resulting maximum emission intensity and total emission area were measured. Calibration graphs were constructed by plotting the maximum emission intensities and areas against the fluoroquinolones concentrations.

Pharmaceutical samples: For the analysis of Catex® 250 (IFC, Spain) and Cunesin 250 (Recordati, Spain), a quantity of a capsule equivalent to 225 mg of the drug was weighted accurately. For the analysis of Amicrobín (Quimifar, Spain) and Esclebin® (Lab. Alacan, Spain), a quantity of a capsule equivalent to 400 mg of the drug was weighted accurately and for the analysis of Surnox 200 (Aventis, Spain) and Oflovir (Vir, Spain), a quantity of a capsule equivalent to 100 mg of the drug was weighted accurately. The drugs were transferred into a 250 ml volumetric flask and diluted to the mark with 5×10^{-3} M sulfuric acid, and diluted twice with 5×10^{-3} M sulphuric acid to obtain final concentrations of the drugs of 15 μg ml^{-1}, 20 μg ml^{-1} and 10 μg ml^{-1} for CIP, NOR and OFL, respectively. For the Ciproxina Simple (Alcon Cusí, Spain), Chibroxin (Thea, Spain), and Exocin® 0.3% (Allergan, Spain), 7 ml in the first case and 0.5 ml in the other two, were transferred into a 25 ml volumetric flask and diluted to the mark with 5×10^{-3} M sulfuric acid and proceeded as described above. The nominal content was calculated from the calibration graphs or regression equations.

Validation of the proposed method

The proposed flow-injection chemiluminescence method, using the manifold depicted in Fig. 2, for the determination of fluoroquinolones, was validated by comparison with a method based on HPLC separation [50], with UV–Vis detection, slightly modified by us.

The chromatographic studies were performed on a Hewlett-Packard Mod 1100 LC instrument, equipped with degasser, quaternary pump, manual six-way injection valve, containing a 20 μl loop, Diode Array spectrophotometer detector and the CHEMSTATION software package to control the instrument, data acquisition and data analysis. An analytical column Nova-Pak C18 (150×3.9 mm, Waters Millipore) was used. A wavelength of 280 nm was selected to monitoring the fluoroquinolones.

The mobile phase was formed by a mixture of solvent A, being a pH 3.0 aqueous formic acid/formate buffer. The buffer concentration was 100 mmol/l. Solvent B, acetonitrile and solvent C, methanol. The mobile phase composition was 75% A, 0% B and 15% of C.

Stock solutions containing 50 μg ml^{-1} of the fluoroquinolones were prepared in methanol. Working fluoroquinolones solutions of different concentrations were prepared by dilution of the stock solution with mobile phase and each sample was injected three times. Linear calibration graphs for area and height against the fluoroquinolones concentration were obtained.

For the analysis of pharmaceutical samples, a pill or capsule was transferred to a 100 ml volumetric flask, dissolved in methanol by shaking and sonicated for 15 min, being the final concentration of 30 μg ml^{-1}, which was filtered through a 22 μm nylon filter. These solutions were diluted with mobile phase to obtain a final concentration of the drug of 5 μg ml^{-1}. This treatment was performed in three pills or capsules of each pharmaceutical formulation, and each sample was injected three times. Ciproxina Simple, Chibroxin and Exocin 0.3% solutions were prepared by adding 1 ml of the pharmaceutical liquid to a 100 ml volumetric flask, and diluting to the mark with methanol, each sample was diluted with mobile phase to obtain a final concentration of 5 μg $ml^{-1}$and filtered through a 22 μm nylon filter and injected three times.

Results and discussion

$Ru(bipy)_3^{2+}$ has proven to be a very sensitive detection system for compounds which contain a secondary or tertiary aliphatic amine [51]. The fluoroquinolones assayed

contain secondary amine (CIP and NOR) or tertiary amine (OFL) since contain a piperazine moiety. Thus, Aly et al. [44] proposed a mechanism, similar to that reported previously for amine determination utilizing its electrogenerated CL reaction with $Ru(bipy)_3^{2+}$ [52]. This mechanism involves the oxidation of $Ru(bipy)_3^{2+}$ and the secondary or tertiary amine present on the fluoroquinolone by Ce (IV). The oxidation product of the amine undergoes deprotonation to form a radical. This reduces the Ru $(bipy)_3^{2+}$ to the excited state, $[Ru(bipy)_3^{2+}]$*, that subsequently emits light returning to $Ru(bipy)_3^{2+}$. This mechanism is in agreement with another reports that suggest that the use of $Ru(bipy)_3^{2+}$ in CL systems produces an orange emission at 610 nm from excited state $[Ru(bipy)_3^{2+}]$* that can be obtained by different reactions which imply electron transfer and regeneration of the $Ru(bipy)_3^{2+}$ specie. One of these it is between the $Ru(bipy)_3^{2+}$ and a reductor [53].

The CL determination of the fluoroquinolones was also studied using potassium permanganate as oxidant. In this case, the CL reaction was slower and the emission intensity smaller. When nitric acid was used to fixing the acid medium, non-reproducible signals were found.

The experimental parameters of the corresponding CL reaction were studied and optimized.

Influence of sulfuric acid concentration on the CL intensity

Ce (IV) salt cannot be dissolved in water at neutral pH; the effect of sulfuric acid concentration, when preparing cerium (IV) salt, was examined to see if this acid had a significant effect on CL emission. As shown in Fig. 3, the CL intensity for CIP and OFL, was maximum and constant at concentrations around 0.5 M. At higher values, the CL intensity decreases. For NOR, the intensity decreased as the sulfuric acid concentration increased. In base of this, an optimum 0.5 M sulfuric acid concentration was chosen.

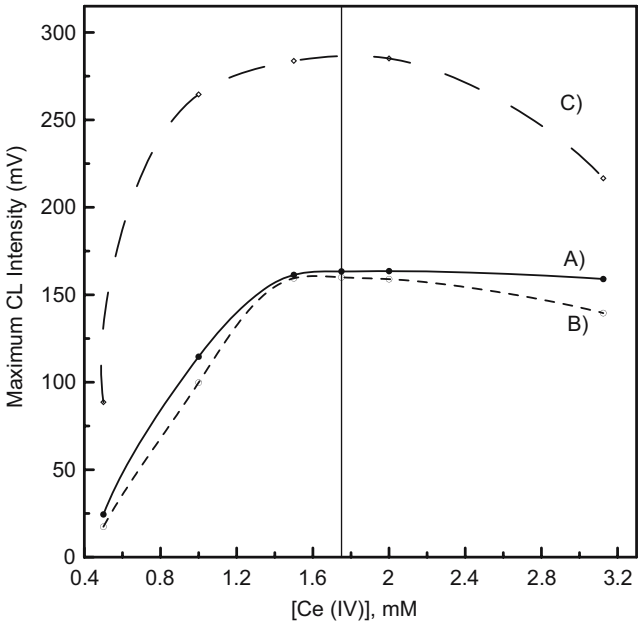

Fig. 4 Effect of Ce (IV)concentration on the maximum CL intensity. H_2SO_4, 0.5 M; $Ru(bipy)_3^{2+}$, 0.5 mM; *A* CIP, 7.0 μg ml^{-1}; *B* NOR, 7.0 μg ml^{-1}; *C* OFL, 1.0 μg ml^{-1}

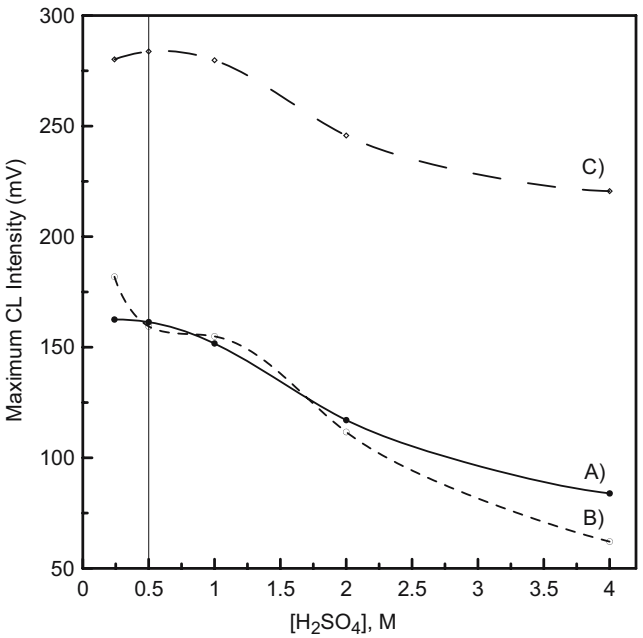

Fig. 3 Effect of H_2SO_4 concentration on the maximum CL intensity. Ce (IV), 1.5 mM; Ru(bipy), 0.5 mM; **A** CIP, 7.0 μg ml^{-1}; **B** NOR, 7.0 μg ml^{-1}; **C** OFL, 1.0 μg ml^{-1}

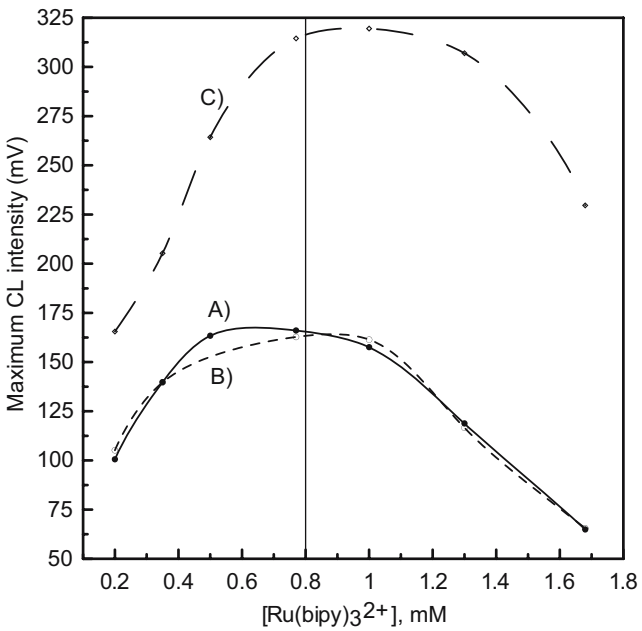

Fig. 5 Effect of $Ru(bipy)_3^{2+}$ concentration on the maximum CL intensity. Ce (IV), 1.75 mM; H_2SO_4, 0.5 M; *A* CIP, 7.0 μg ml^{-1}; *B* NOR, 7.0 μg ml^{-1}; *C* OFL, 1.0 μg ml^{-1}

Table 1 Analytical and statistical parameters for the determination of fluoroquinolones with the proposed method

	CIP	NOR	OFL
Linear range (μg ml^{-1})	1.3–30	1.4–43	0.7–15
Correlation coefficient	0.999	0.999	0.998
Linearity (%)	99	99	99
Sensitivity, γ^{-1} (μg ml^{-1})	0.43	0.47	0.20
LOD[a] (μg ml^{-1})	0.96	1.05	0.48
LOD[b] (μg ml^{-1})	0.40	0.42	0.21

[a] Clayton et al. ($\alpha=\beta=0.05$) [60]
[b] Long and Winefordner ($k=3$) [61]

Influence of the Ce (IV) concentration on CL intensity

The concentration of Ce (IV) upon the CL behaviour of fluoroquinolones was examined over the range 0.5–3.1 mM in 0.5 M sulfuric acid. The results are shown in Fig. 4. Maximum and constant emission intensity was obtained since 1.5 mM Ce (IV). Therefore, 1.75 mM Ce (IV) was adopted as the working solution in future experiments.

Influence of the $Ru(bipy)_3^{2+}$ concentration on CL intensity

The study of the influence of $Ru(bipy)_3^{2+}$ concentration, over the range 0.2–1.7 mM, Fig. 5, shows that the maximum CL emission first increased with $Ru(bipy)_3^{2+}$ concentration until a value (0.8 mM) where it is constant. A higher concentration than 1.15 mM is not recommended because the CL emission decreases. A concentration of 0.8 mM (within the maximum and constant range) was selected.

Influence of flow rate on CL intensity

The flow rate is an important parameter in CL detection because the time taken to transfer the excited product into the flow cell is critical for maximum collection of the emitted light [54]. Too low or too high flow rates result in a decrease or even absence of CL in the flow cell. The optimum flow rate was found to be 10.5 ml/min.

Analytical performance

The above-described system and optimum experimental conditions (1.75 mM Ce (IV) in H_2SO_4 0.5 M and Ru $(bipy)_3^{2+}$ 0.8 mM) were used to determine the fluoroquinolones by direct measurement of the CL transient signal, using the peak height (maximum emission intensity). Linear calibration graphs for each CL signal parameter against the fluoroquinolone concentration were obtained over the range of 1.3–30, 1.4–43 and 0.7–15 μg ml^{-1} for CIP, NOR and OFL respectively, using eight standards and three replicates per point (Table 1).

Applications and study of interferences

To evaluate the possible analytical applications on the CL method described above, the effect of some common excipients (citrate, manithol, lactose, sucrose, glucose, starch) used in pharmaceutical preparations was studied. The procedure consists of preparing different synthetic solutions, each one containing the fluoroquinolone (15, 20 and 7.0 μg mL^{-1} of CIP, NOR and OFL, respectively) and one excipient, in proportion 50:1, *w*:*w*, (substance/fluoroquinolone). Later, it was measured the CL signal of these solutions using the conditions fixed and no interference was observed from these recipients (Table 2).

The proposed method was satisfactorily applied to the analyses of the fluoroquinolones in the Spanish pharmaceutical products that contain these drugs in different dosage. The assay results, expressed as a percentage of the nominal contents, resulting from the average of three determinations, are summarized in Table 3, where it is observed that the recoveries are all close to 100% and the precision is satisfactory. The results were validated by an HPLC method, as described under procedure.

Table 2 Effects of various excipients on the determination of CIP (15 μg ml^{-1}), NOR (20 μg ml^{-1}) and OFL (7.0 μg ml^{-1})

Excipient/fluoroquinolone 50:1	Recovery (%±SD)		
	CIP	NOR	OFL
Citrate	101±2	101±1	100±2
Manithol	103±3	101±1	98±3
Lactose	102±4	98±1	99±2
Sucrose	102±4	101±2	98±2
Glucose	104±3	99±1	103±1
Starch	98±6	98±2	99±2

Table 3 Recoveries in the determination of fluoroquinolones in commercial formulations

Fluoroquinolone	Commercial formulations	Fluoroquinolone nominal content	Recovery (%±SD)		
			CL method	HPLC method	
				Height	Area
CIP	Ciproxina simple	3 mg/ml	102±3	103±1	97±1
	Catex	250 mg/tablet	101±1	101±7	98±4
	Cunesin	250 mg/tablet	102±1	103±4	98±3
NOR	Amicrobín	400 mg/tablet	102±1	96±1	99±1
	Esclebin	400 mg/tablet	100±1	93±1	97±1
	Chibroxin	3 mg/ml	99±1	96±1	100±1
OFL	Exocin, 0.3%	3 mg/ml	100±1	102±1	103±1
	Surnox 200	200 mg/tablet	103±2	96±1	96±1
	Oflovir	200 mg/tablet	101±1	101±1	102±1

In order to compare the results obtained by using the two methods, lineal regression analysis of found concentration values for the two methods was applied. Regression was performed using concentration data for the three analytes simultaneously, taken from all the commercial formulations reported in Table 3, as recommended in literature [55] in order to obtain better estimates of the experimental errors. The estimated intercept and slope ($\hat{a}$ and $\hat{b}$, respectively) were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test [56]. The fitted regression parameters were: slope, 0.9979; intercept, −0.1004, with the theoretical (a=0, b=1) point being included within the corresponding ellipse and we concluded that there was no significant difference between the results obtained by using the two methods.

Simultaneous determination of binary fluoroquinolones mixtures

With the aim of performing the analysis of binary mixtures of ofloxacin and ciprofloxacin and of ofloxacin and norfloxacin, a chemometric approach, based in partial least squares (PLS-1) was evaluated. The independent calibration curves for each component were used to establish the analytical range of concentration and to study the additivity

Table 4 Composition of the calibration set composed of the samples of calibration (1–13) and prediction (14–18) sets

Sample number	Binary mixtures (μg ml^{-1})			
	[OFL]	[CIP]	[OFL]	[NOR]
Samples of the calibration set				
1	2.19	4.44	2.92	5.85
2	7.50	4.44	9.94	5.85
3	12.8	4.44	17.0	5.85
4	2.19	14.9	17.0	20.0
5	7.50	14.9	9.94	20.0
6	12.8	14.9	17.0	20.0
7	2.19	25.7	2.92	34.1
8	7.50	25.7	9.94	34.1
9	12.8	25.7	17.0	34.1
10	0	14.9	0	20.0
11	14.9	14.9	20.3	20.0
12	7.50	0	9.94	0
13	7.50	29.7	9.94	40.1
Samples of the prediction set				
14	10.6	3.61	15.2	16.0
15	5.78	16.5	4.06	24.0
16	8.97	11.7	16.8	38.9
17	3.59	24.2	9.53	38.9
18	15.0	27.0	3.65	17.2

of the time-resolved chemiluminescence signals of the binary mixtures of the components.

The PLS method involves a calibration step in which, the relation between bi-dimensional time-resolved chemiluminescence and analyte concentrations, is estimated from a set of reference samples (calibration set), and a prediction step in which the results of the calibration are used to estimate the component concentrations in unknown samples (prediction set).

All calculations were done using Matlab 5.3. The routine for PLS-1 follows the classical PLS algorithm and a useful Matlab graphical interface was used for easy data manipulation and graphic presentation [57]. The interface provides a simple means of loading the data matrices into the Matlab working space before running PLS-1.

Calibration and prediction sets

A 13-samples set was built to perform the calibration process with the PLS-1 method. The calibration corresponds to a central composite design composed of a two components full-factorial design at three levels, combined with a star design. Similarity, with the aim of validating the chemometric proposed method, a prediction set of binary samples was prepared. The analyte concentrations were comprised in the calibration set range. The compositions of the calibration and prediction sets are shown in Table 4.

Optimization of the PLS model

In the Fig. 6, a representation of the time-resolved chemiluminescence signals for binary mixtures of ofloxacin plus ciprofloxacin (a), and ofloxacin plus norfloxacin (b), are shown. The differences between the time-resolved chemiluminescence profile of ofloxacin respect to ciprofloxacin or norfloxacin are evident in the representation, and the base for the binary mixture chemometric resolution procedure.

The time interval to record the chemiluminescence response was optimized for each component of the corresponding binary mixture, to obtain the maximum coefficient of correlation in the calibration step. The time windows selected for the first mixture were between 0 and 0.138 min for ofloxacin determination and between 0.138 and 1.496 min for ciprofloxacin determination, and for the second mixture were between 0 and 0.199 min for ofloxacin determination and between 0.178 and 0.597 min for norfloxacin determination, being the resolution 0.001 min.

In order to determine the correct number of loading vectors to be used for the modeling of the data, a cross-validation calculation for all samples in the calibration set was performed to calculate the PRESS (prediction residual error sum of squares) [58, 59]. An optimum number of loading vectors of two was found for all the cases.

The PLS model was applied to the data set of problem samples (prediction set of Table 4). The samples analyzed were composed by binary mixtures of variable amounts of the components randomly selected. The recovery values obtained in the analysis of the data set are summarized in Table 5. The recoveries obtained are indicating a satisfactory resolution of the binary mixtures investigated.

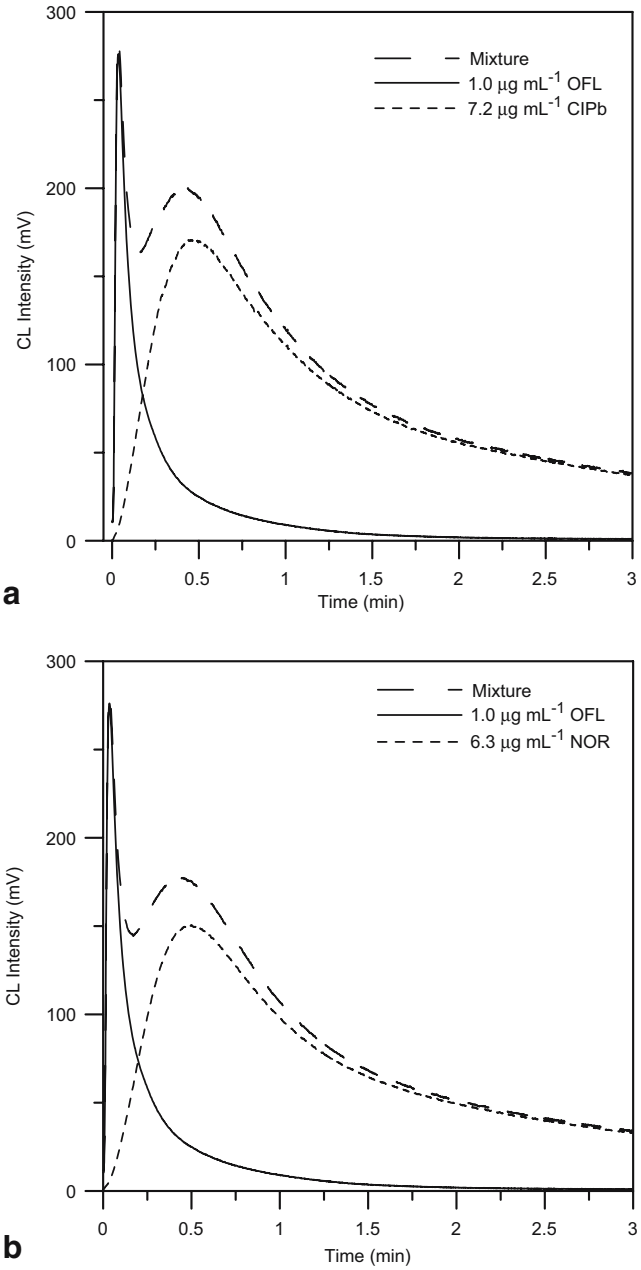

Fig. 6 Time-resolved chemiluminescence signals for binary mixtures of ofloxacin plus ciprofloxacin (**a**) and ofloxacin plus norfloxacin (**b**)

Table 5 Recovery values obtained in the analysis of the prediction sets

	Actual ($\mu g\ ml^{-1}$)	Predicted ($\mu g\ ml^{-1}$)	%*R*	Statistical parameters		
				RMSEP	REP %	Factors
Binary mixture CIP–OFL						
Ciprofloxacin	3.60	4.43	123	1.50	9.0	2
	16.47	13.64	83			
	11.67	13.04	112			
	24.25	23.53	97			
	26.998	26.57	98			
			103±15[a]			
Ofloxacin	10.57	10.09	95	0.42	4.8	2
	5.78	6.35	110			
	8.97	9.39	105			
	3.59	3.74	104			
	15.01	15.37	102			
			103±5[a]			
Binary mixture NOR–OFL						
Norfloxacin	16.03	17.34	108	2.13	8.9	2
	24.05	22.01	92			
	38.88	34.81	90			
	23.25	23.07	99			
	17.23	17.68	103			
			98±7[a]			
Ofloxacin	15.21	14.43	95	0.43	5.1	2
	4.06	3.83	95			
	9.53	9.62	101			
	9.53	9.75	102			
	3.65	3.19	87			
			96±6[a]			

[a] Average recovery values correspond to the recoveries of the five prediction samples ± the computed standard deviations.
RMSEP Root mean square error of prediction and *REP %* relative error of prediction

Conclusions

The chemiluminescent reaction between fluoroquinolones and Ce (IV) in the presence of tris(2,2′-bipyridyl) ruthenium(II), was studied using the stopped-flow technique in a continuous-flow system and a simple, rapid and highly sensitive chemiluminometric method is described for the determination of fluoroquinolones in dosage forms. Furthermore, as it has been described, no interference from common excipients in commercial preparations is observed and the results obtained were in agreement well with those given by a HPLC method, indicating that the present method has potential for the analysis of these fluoroquinolones in drugs. The method does not require sophisticated instrument and could be widely used in the routine fluoroquinolones quality control in the analysis of pharmaceutical preparations.

As far as we know, this is the first time that the stopped-flow time-resolved chemiluminescence signals are proposed and combined with first-order multivariate calibration methods for mixture resolution.

A satisfactory resolution capacity was obtained by the application of a first-order multivariate method in the analysis of binary mixtures of ofloxacin–ciprofloxacin and ofloxacin–norfloxacin. It has been shown that the utilization of time-resolved chemiluminescence signals is a viable way to increase the information useful and disposable for a calibration model.

In our study, the differences in the time-resolved chemiluminescence signals of the investigated binary mixtures were used to resolve the mixtures, applying partial least squares multivariate calibration. This approach opens a new possibility of improvement of chemiluminescence methods by combining with different chemometric approaches.

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