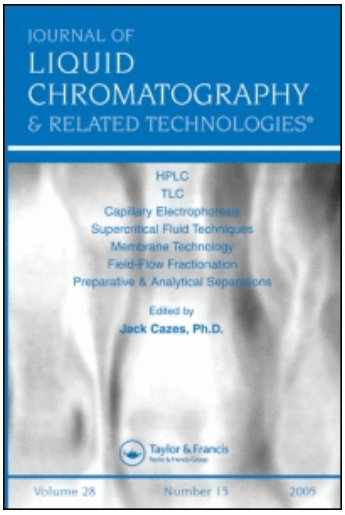

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Abstract: A capillary electrophoretic method for the separation of the enantiomers of ofloxacin using carboxymethyl-β-cyclodextrin (CM-β-CD) as chiral selector is described. The effect of the type of cyclodextrin and its concentration, buffer concentration, and its pH, as well as instrumental parameters, such as applied voltage and temperature were systematically studied. The highest resolution of ofloxacin enantiomers obtained was around 2.8. This was achieved using Tris-citrate buffer (pH 4.5) that contained 3 mg mL^{-1} CM-β-CD and using UV detection (254 nm), applied voltage (12 kV), and capillary temperature of 25°C. Acceptable validation criteria for selectivity, precision, linearity, limit of detection, and quantitation were also included. Recoveries between 98.3–103.4% were obtained when the method was used to determine the enantiomers of ofloxacin that were spiked to placebos. The proposed method is fast, sensitive, inexpensive, and its usefulness was demonstrated for the analysis of five pharmaceutical preparations, two of which just contained the S-ofloxacin while the other three contained both isomers as racemic mixtures.

Keywords: Ofloxacin, Enantiomers, Capillary electrophoresis, Pharmaceutical formulations

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INTRODUCTION

Racemic ofloxacin, having the chemical name (±)-9-fluro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo7H-pyrido[1,2,3-de]-1,4-benzooxazine-6-carboxylic acid (Figure 1), belongs to the quinoline class of antibiotics. It has been shown that the antibacterial activity of the levo-enantiomer of ofloxacin (S-ofloxacin or levofloxacin) is 8-128 times higher than that of the R-enantiomer and twice as potent as that of the racemate.[1,2] Currently, the drug is either marketed as a racemic mixture, i.e., consisting of equal amounts of R- and S-enantiomers or only the S-isomer. Therefore, research activities on the separation of racemic ofloxacin and its application in pharmaceutical formulations are important.

Analytical methods that have been reported for the quantitative determination of racemic ofloxacin in pharmaceutical preparations include HPLC[3] and CE.[4,5] However, none of these methods were able to discern the quantities of the individual enantiomers of ofloxacin in pharmaceutical preparations, which require different strategies for their separation.

Chiral separation of ofloxacin in biological fluids has been achieved by HPLC, either by the addition of chiral selector to the mobile phase or using chiral stationary phase.[6–8] However, disadvantages of these methods include tedious sample preparation procedures involving extraction and/or derivatization steps, and the relatively expensive chiral columns that were required.

Over the last two decades, CE has proven to be a powerful alternative to HPLC for enantioselective analysis.[9–13] Compared to HPLC, CE offers several distinct advantages including simplicity, short analysis times, high efficiencies, low consumption of chiral selector (translated to reduced running cost), and different separation mechanisms. The most frequently used chiral selectors in CE are cyclodextrins (CDs) and their derivatives.[14,15] They have negligible UV absorbance, are water-soluble, and are commercially available in a large number of modified forms. The enantioselective recognition results from the inclusion of a hydrophobic portion of the analyte in the CD cavity and also from hydrogen bonding to chiral hydroxyl moieties.

A number of CE methods have been proposed for the qualitative chiral separation of ofloxacin enantiomers using different chiral selectors such as cyclodextrins (CDs),[16–19] vancomycin,[20] bovine serum albumin,[21,22] or a combination of γ-cyclodextrin (γ-CD) and D-phenylalanine and zinc

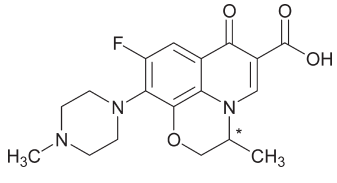

Figure 1. Chemical structure of ofloxacin; asterisk indicate the chiral centre.

sulphate.[23] In some of these studies, the resolution factors were poor,[18,20] and often lack reproducibility and are not practicable due to the long migration times.[18,20] The quantitative assay of ofloxacin enantiomers and it metabolites in human urine using CE with laser induced fluorescence detection has been reported.[24] However, laser induced fluorescence detectors are expensive and are, thus, not widely used. The quantification of ofloxacin enantiomers in Hank's Balanced Salt Solution (HBSS) as physiological solution for pharmacokinetic studies has also been optimized.[25] To the best of our knowledge, no CE method for the quantitative chiral analyses of ofloxacin enantiomers in pharmaceutical formulation has been published.

In this study, a simple method for the enantiomeric separation of ofloxacin, using a low concentration of carboxymethyl-β-cyclodextrin (CM-β-CD) in the normal polarity mode is described. Ionizable β-CD derivatives, and in particular those containing carboxy groups, such as carboxymethyl[26–28] have been used widely as chiral additives in CE. They often provide higher flexibility in the optimization of enantiomeric resolution, mainly due to the possibility of changing its charge by altering the pH of the background electrolyte, which directly affects the electrophoretic mobility. Finally, the developed method was applied to the quality control of pharmaceutical formulations containing R- and S-ofloxacin.

EXPERIMENTAL

Chemicals and Reagents

Ofloxacin, ($\pm$)-9-fluro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo7H-pyrido[1,2,3-de]-1,4-benzooxazine-6-carboxylic acid, S-ofloxacin and tris(hydroxymethyl)aminomethane were purchased from Sigma-Aldrich (St. Louis, USA). β-cyclodextrin (β-CD), α-cyclodextrin (α-CD), 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-α-cyclodextrin (HP-α-CD), 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD), and carboxymethyl-β-cyclodextrin (CM-β-CD), were obtained from Fluka (Buchs, Switzerland). Anhydrous citric acid, was purchased from Sigma (St. Louis, USA). Commercial pharmaceutical preparations in the form of tablets, (claimed to contain 500 mg, 200 mg, and 100 mg active ingredient) were purchased from the campus drug store and were manufactured by different pharmaceutical companies. Milli-Q water was used for the preparation of all solutions.

Instrumentation and Electrophoretic Conditions

The analysis was carried out on a Waters Capillary Ion Analyzer (Milford, MA, USA) which was interfaced to a Waters PC 800 Workstation. Uncoated fused-silica capillary (total length, 35 cm and internal diameter,

50 μm) were used for the enantiomeric separation. The separation was carried out at 25°C and applying a voltage of 12 kV. Samples were injected hydrostatically for 25 seconds. Detection was achieved at 254 nm.

A new uncoated fused silica capillary was conditioned by flushing with 1 M NaOH for 30 minutes, then 0.1 M NaOH for 10 minutes, and water and buffer each for 15 minutes. The running buffer consisted of 50 mM citric acid that had been adjusted to the desired pH with 1 M Tris solution. The running buffer solution was passed through a 0.2 μm cellulose nitrate membrane filter (Whatman International, Maidstone, England) and was degassed by sonication prior to use.

Prior to each analysis, the capillary column was rinsed with 0.1 M NaOH for one minute, and then purified water, followed by the carrier electrolyte, each for 2 minutes between the runs.

Stock and Standard Solutions

Standard stock solution (1000 μg mL^{-1}) of racemic ofloxacin and S-ofloxacin were prepared in 0.1 M NaOH and was kept refrigerated. Working standard solutions were prepared daily by diluting suitable aliquots of the stock solution with water. The standard solutions were stored in brown glass vials for protection from light.

Pharmaceutical Sample Preparation

Five tablets were weighed, ground, and mixed in a mortar. An appropriate amount of the powder was taken and dissolved in 25 mL of 0.1 M sodium hydroxide by ultrasonic for three minutes and diluted to 100 mL with water. The sample was filtered through a membrane (0.22 μm) and 1 mL of the filtrate was diluted with water to 10 mL. This solution was introduced to the CE system for the separation.

RESULTS AND DISCUSSION

Optimization of Separation Conditions

Choice of Chiral Selector

A previous study on the chiral separation of ofloxacin indicated that the electrophoretic separation was possible at nearly all pH values[24] except at their isoelectric points which is 7.4 for ofloxacin.[29] So pH 4.0 was chosen for preliminary studies. In an attempt to find a suitable and readily available chiral selector, the chiral separation of the enantiomers was attempted using

different cyclodextrins, namely native α-CD, β-CD, HP-α-CD, HP-β-CD, HP-γ-CD, and CM-β-CD. No chiral recognition was observed with α-CD, β-CD, and HP-γ-CD, whereas the use of HP-α-CD, HP-β-CD, and CM-β-CD were feasible. CM-β-CD seems to be the best candidate and was thus used in further studies. Wang et al.[19] described the separation of basic drugs including ofloxacin using CM-β-CD as chiral selector. The separation of ofloxacin enantiomers using methyl-β-cyclodextrin and sulfobutyl-β-cyclodextrin were reported by Awadallah et al.[25] and Horstkotter et al.,[24] respectively.

Choice of pH

pH is an important parameter to be optimized as it affects the ionization of the silanol group of the capillary wall, which in turn affects the magnitude of the electroosmotic flow (EOF). Furthermore, for pH value lower than its pKa, both enantiomers migrate as cations in the electrophoretic system. The pKa values for ofloxacin have been reported as 8.0, and 6.0 for the basic and acidic group, respectively.[18]

The effect of pH on the resolution (Rs) and migration time was investigated over the pH range 3.0–7.0, using 50 mM buffer solutions prepared at different pH containing 3.0 mg mL^{-1} CM-β-CD. The results are shown in Figure 2. Increase in resolution was observed when the pH values were increased from 3.0 to 4.5 and then decreased from pH 5.0 to 7.0 and was totally unresolved at pH 7.0. At pH 4.0, CM-β-CD behaves as a neutral selector since it is unionized. At low pH, the ofloxacin molecules are protonated and are positively charged and, thus, do not interact with CM-β-CD. As the pH is increased from 3 to 4.5 CM-β-CD becomes anionic and this increases the interaction between

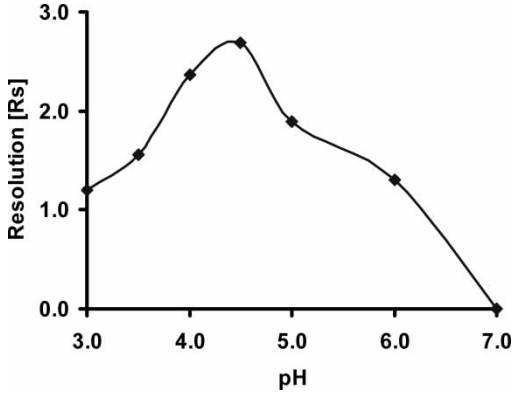

Figure 2. Effect of pH on the resolution of ofloxacin enantiomer (buffer concentration 50 mM; CM-β-CD concentration, 3 mg mL^{-1}; temperature, 25°C, and applied voltage, 12 kV).

CM-β-CD and ofloxacin. The drop in the resolution from 4.5 to 7.0 is a result of repulsion between CM-β-CD zwitterionic ofloxacin. Another reason for the final drop in resolution could be the increased EOF in the capillary and the decreased separation windows as a result of more robust EOF.[27] Consequently, better resolution was obtained at pH 4.5.

Choice of Buffer Concentration

The concentration of the buffer may also play a significant role in the chiral separation of drugs. In this work, the effects of buffer concentration (25–100 mM) were investigated, by maintaining 3 mg mL^{-1} CM-β-CD in the background electrolyte (BGE) at pH 4.5. As the buffer concentration increased, the resolution slightly decreased (data not shown). This may be attributed to the increase of current generation and Joule heating. A 50 mM buffer was selected to achieve good resolution without sacrificing the migration time.

Choice of Cyclodextrin Concentration

CD concentration is an essential parameter in the optimization of chiral separations.[30,31] The effects of different concentrations of CM-β-CD (1–4 mg mL^{-1}) on the resolution and migration times were investigated. Chiral separation was achieved for all concentrations tested. Increasing the concentration of CM-β-CD causes the resolution and the migration time (not shown but to a lesser extent) to increase (Figure 3). This is probably due to favourable complexation with the chiral selector under these conditions. In the present work, it was found that CM-β-CD exhibits high resolution even when low concentration of a chiral selector was used. The high enantioselectivity of CM-β-CD is probably, at least partly, related to deprotonation of the carboxyl groups at pH 4.5. The cationic compounds will more strongly interact with

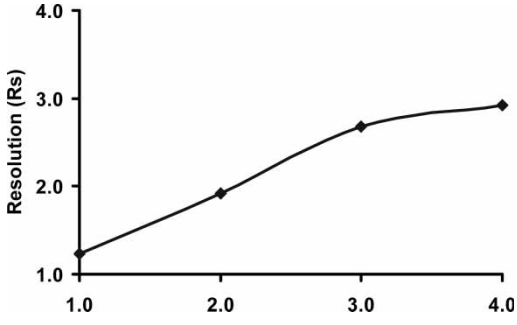

Figure 3. Effect of CM-β-CD concentration on the resolution of ofloxacin enantiomers (buffer concentration 50 mM, pH 4.5, temperature, 25°C, and applied voltage, 12 kV).

the chiral selector and also CM-β-CD will migrate towards the anode, resulting in significant differences in the electrophoretic mobility between the free and the complexes form of the enantiomers.[27,32] CM-β-CD (3 mg mL^{-1}) was chosen as a compromise between resolution and speed of analysis.

Choice of Applied Voltage

The effect of applied voltage was also investigated, as it is well known that increasing the voltage gives rise to shorter migration times.[33] However, the generation of Joule heat may limit the theoretical gain in the resolution and efficiency when the voltage is increased. In this study, voltages between 8 and 20 kV were investigated. Resolution with good peak shape and reasonable migration time was found when operated at 12 kV.

Choice of Injection Time

In order to further reduce the detection limits, the injection time was varied from 15–35 seconds. Using hydrostatic injection, 25 seconds injection time was selected as the optimal value.

Details of the optimized electrophoretic separation conditions are summarized in Table 1, while Figure 4 shows a typical electropherogram obtained when a standard solution of 100 $\mu g\ mL^{-1}$ of racemic ofloxacin were injected under the optimized conditions.

Method Validation

Selectivity

No interference from the formulation excipients could be observed at the migration times of the enantiomers. The S-enantiomer was eluted first and this was confirmed by spiking the S-enantiomer standards.

Table 1. Optimum CE operating conditions

Electrolyte	50 mM Tris-citrate buffer, pH 4.5, 3 mg mL^{-1}, CM-β-cyclodextrin
Applied voltage	12 kV
Sample injection	Hydrostatic, 25 s
Capillary temperature	25°C
Fused silica capillary	35 cm total length (27.5 cm effective length) × 50 μm i.d.
Detection wavelength	254 nm

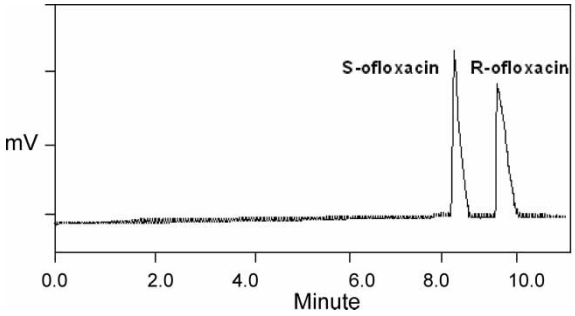

Figure 4. Electropherogram obtained from the injection of 100 μg mL^{-1} standard racemic ofloxacin. Please refer to Table 1 for CE conditions.

Precision

Intraday precision was assessed by injecting racemic ofloxacin standards at three different concentrations (50, 150, and 250 μg mL^{-1}) six times. In all cases, the relative standard deviation (RSD) for migration times and corrected peak areas were less than 1.5 and 4.0, respectively (Table 2). The interday precision was assessed with three concentrations of the standard (50, 150, and 250 μg mL^{-1}) injected six times for three consecutive days. In all cases, good precision as evidenced from the RSD for migration time and corrected peak areas of less than 5.0 and 4.0, respectively, were found (Table 2).

Table 2. Within-day and interday reproducibility for the repeated injection of different concentrations of racemic ofloxacin standards

	RSD (%)		RSD (%)	
	Migration time		Corrected peak areas	
Conc. (μg mL^{-1})	S-ofloxacin	R-ofloxacin	S-ofloxacin	R-ofloxacin
Intraday precision (n = 6)				
50	1.29	1.41	3.67	3.44
150	0.31	0.33	1.98	0.76
250	1.49	1.36	3.02	2.41
Interday precision (n = 18)				
50	4.17	4.92	3.78	3.27
150	3.15	3.47	2.25	1.86
250	3.67	4.18	3.75	3.64

Accuracy

The accuracy of the method was evaluated by conducting recovery studies on placebos. Several aliquots of the racemic ofloxacin at three different concentrations (50, 150, and 250 $\mu g\ mL^{-1}$) were added to an analytical placebo prepared from the excipients. The results are summarized in Table 3. Recoveries between 98.36 and 103.38% were found in all cases.

Linearity

The calibration graph was constructed by plotting corrected peak areas as a function of analytes concentration in $\mu g\ mL^{-1}$. Six standard solutions containing 25 to 250 $\mu g\ mL^{-1}$ racemic ofloxacin were injected. The linear regression equations obtained are summarized below:

$$\text{S-ofloxacin: } y = 37.59x - 7.53, \quad r^2 = 0.9983$$

$$\text{R-ofloxacin: } y = 34.93x - 5.65, \quad r^2 = 0.9981$$

Limit of Detection (LODs) and Quantitation (LOQs)

The limit of detection of S-ofloxacin and R-ofloxacin enantiomers were 3.7 $\mu g\ mL^{-1}$ for both enantiomers. This was obtained by multiplying the standard deviation by 3. The limits of quantitation, estimated by multiplying the standard deviation by 10, were 7.47 $\mu g\ mL^{-1}$ and 7.31 $\mu g\ mL^{-1}$ for S- and R-ofloxacin, respectively.

Analysis of Pharmaceutical Formulations

The developed method was applied for the analysis of ofloxacin in different commercial pharmaceutical formulations. Triplicate determinations were carried out. The results obtained are summarized in Table 4. It is clear that some

Table 3. Recoveries obtained from the determination of ofloxacin in placebos that contained different levels of spiked standards

Sample no.	Racemic ofloxacin ($\mu g\ mL^{-1}$) spiked	Recovery (%, mean ± S.D.)	
		S-ofloxacin	R-ofloxacin
1	50	98.36 ± 1.72	99.28 ± 1.45
2	150	103.4 ± 3.52	101.2 ± 0.21
3	250	102.9 ± 1.08	99.45 ± 0.55

Table 4. Results for the determination of pharmaceutical formulations containing racemic ofloxacin or S-ofloxacin

Sample no.	Commercial formulation	S-ofloxacin (mg)	R-ofloxacin (mg)	Total active ingredient (mg)
1	PRIFLOXCIN (200 mg)	100.7 ± 3.29	99.22 ± 0.41	199.9 ± 3.70
2	OFLOX (200 mg)	102.3 ± 2.39	104.6 ± 1.51	206.8 ± 3.90
3	TARIVID (100 mg)	53.11 ± 0.96	51.32 ± 1.12	104.4 ± 2.08
4	TAVANIC (500 mg)	501.4 ± 4.40	0	501.4 ± 4.40
5	LOXOF (500 mg)	496.5 ± 0.79	0	496.5 ± 0.79

Figures in bracket denote the manufacturer's claimed value.

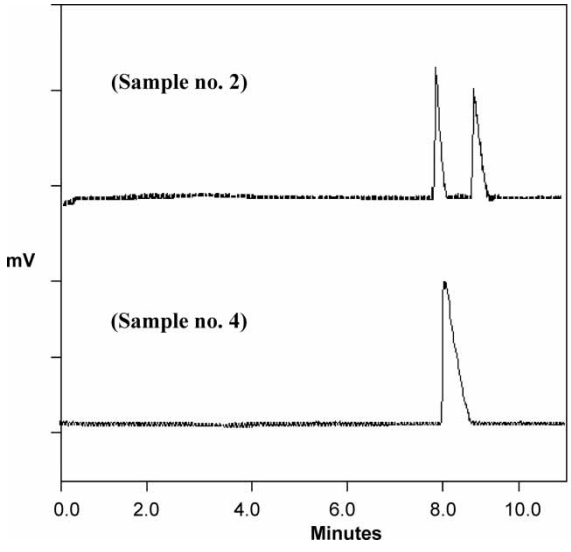

Figure 5. Electropherograms of two analyzed pharmaceutical formulations sample No. (2) and (4). Please refer to Table 1 for CE conditions.

samples were prepared as racemic mixtures (samples 1–3), while samples 4 and 5 contain a single active ingredient, i.e., S-ofloxacin. In all cases, there were good agreements between the total values as claimed by the manufacturers. Figure 5 shows typical electropherograms of these pharmaceutical formulations.

CONCLUSIONS

A simple, cheap, and rapid CE method with normal polarity mode, and UV-detection was developed for the chiral separation of ofloxacin

enantiomers using CM-β-CD as a chiral selector. Several experimental parameters that affect the chiral separation were investigated. The optimum CE conditions were: 50 mM Tris-citrate (pH 4.5) containing 3 mg mL^{-1} CM-β-CD as BGE; applied voltage, 12 kV; capillary temperature, 25°C, and injection time, 25 seconds. Good analytical performance with regards to linearity, repeatability, reproducibility, and accuracy was achieved. Although the separation of enantiomers of ofloxacin had been reported, none were used for the determination of the enantiomers in pharmaceutical formulations. The present method is superior over the reported CE methods in terms of faster analysis time (under 10 min) compared to the report of Wang et al.[19] (18 min) and Horstkkotter and Blaschke[24] (15 min). Another notable feature of the proposed method is the use of lower concentrations of chiral selectors (3 mg mL^{-1} versus 15.6–40 mg mL^{-1}.[19,25] The proposed method is, therefore, recommended to be adopted as quality control protocol in pharmaceutical industries.

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