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Quantitation of the enantiomers of ofloxacin by capillary electrophoresis in the parts per billion concentration range for in vitro drug absorption studies

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Abstract

Ofloxacin, a chiral fluoroquinolone, possesses two optical isomers. The antibacterial activity of S-(−)-ofloxacin is reported to be 8–128 times higher than that of R-(+)-ofloxacin. A capillary zone electrophoresis method has been developed to quantify the enantiomers of ofloxacin in high diluted samples (20–700 ng/ml for each enantiomer). After fluid–fluid extraction of ofloxacin from physiological solution electrokinetic injection was employed to improve the sensitivity. The method was optimised using a central composite design. Four experimental factors were investigated: the background electrolyte concentration, the methyl-β-cyclodextrin concentration, the buffer pH and the temperature. The amount migrated into the capillary, determined by the peak area, the resolution between the ofloxacin enantiomers, the migration time and the generated current were evaluated as responses. The quantification limit is 11.4 ng/ml for S-ofloxacin and 10.8 ng/ml for R-ofloxacin. The method has shown good validation data in terms of precision and recovery rate.
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1. Introduction

Ofloxacin, (±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (Fig. 1), is a totally synthetic fluoroquinolone with gyrase-inhibiting action in bacteria. Differences in the antibacterial activity of the isomers of fluoroquinolones are well known. The antibacterial activity of the S-(−)-
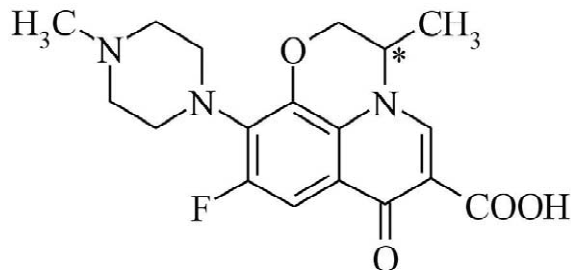

Fig. 1. Molecular structure of ofloxacin; *, chiral centre.

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isomer is 8–128 times higher than that of the R-(+)-isomer [1,2]. Ofloxacin shows a saturable and stereoselective intestinal secretion of its enantiomers in the rat [3]. The elimination process favours the R-(+) form of the molecule.

Today, regulatory authorities in the USA, Europe, China and Japan provide guidelines indicating that preferably only the active enantiomer (eutomer) of a chiral drug should be brought to the market. Capillary electrophoresis (CE) is a powerful method used for enantioseparation. Especially for biological and physiological fluids the CE offers several advantages because only small amounts of sample are required. Many reviews list applications of the CE for analysis of drugs in biological fluids [4–7]. If the drug concentration in the physiological fluids is in the higher $\mu g/ml$ range, direct injection into the capillary is possible [8]. Several CE methods have been proposed for the enantioresolution of racemic ofloxacin using different chiral selectors [9–17]. Because of the lack of detection sensitivity, none of the indicated methods provides the necessary quantitation range and are thus not applicable in our case. In comparison to HPLC the sample volume applied in CE is about 1000-fold smaller and the detector cell path length is 100 times less than in HPLC. Therefore, CE with UV detection is in general not sensitive enough to quantify very low amounts of drugs in physiological fluids. Several approaches are reported to improve the sensitivity of the CE. They can be divided into two: increasing the amount loaded into the capillary and improving the sensitivity at the detector site [18,19]. The amount of analytes loaded into the capillary can be increased by increasing the injection load and volume using sample stacking, isotachophoresis or electrokinetic injection. This leads to a pronounced increase in sensitivity. The use of wide-bore capillaries or modified capillaries is the other way to overcome this weakness of the CE. In addition, the use of alternative detection techniques to the UV detection like laser-induced fluorescence detection (LIF) or mass spectroscopy (MS) can further increase the sensitivity of CE. In the case of ofloxacin the use of the LIF detection to improve the sensitivity has been reported [9]. This method, however, provided a detection limit of only 250 ng/ml for ofloxacin enantiomers. Laser-induced fluorescence detectors are very expensive and not widespread.

The aim of the current work was to optimise a method to quantify the enantiomers of ofloxacin in the ng/ml range in Hank's balanced salt solution (HBSS) as physiological solution—for pharmacokinetic studies of ofloxacin by Caco-2 cells as absorption model—by CE using UV detection. Methyl-β-cyclodextrin in the running buffer was used as chiral selector. Therefore, two different methods were evaluated to quantify the isomers of ofloxacin at different concentration ranges. A statistical experimental design approach was carried out in order to determine optimal conditions for high resolution in a short time with high sensitivity and to set up a robust method.

2. Experimental

2.1. Materials

Racemic ofloxacin (batch No. D006) and S-(−)-ofloxacin hemihydrate (Ch.B. C106) were kindly donated by Aventis Pharma (Bad Soden, Germany). Procaine hydrochloride (internal standard, I.S.) was obtained from Fluka (Buchs, Switzerland). Enrofloxacin (P. No. 317715K) as I.S. was kindly provided by Bayer (Monheim, Germany) and methyl β-cyclodextrin [β-CD; Cavasol W7 M Pharma, degree of substitution per anhydro glucose unit (SD): 1.7–1.9] by Wacker (Munich, Germany). Sodium dihydrogenphosphate dihydrate (Ch.B. K22700745), orthophosphoric acid 85% (P. No. 100573), dichloromethane, methanol and Titrisol (NaOH) were supplied by Merck (Bruchsal, Germany). Water used for standard and sample preparation was obtained by distillation after deionisation by reverse osmosis. The electrolyte solutions were filtered through a 0.2 μm membrane filter (Sartorius, Göttingen, Germany) before use.

2.2. Instrumentation and electrophoretic procedure

Experiments were carried out on a P/ACE system 5500 instrument (Beckmann Coulter, Unterschleissheim, Germany) equipped with a 30 kV power supply and an UV spectrophotometric detector connected to a data collection system. The system is

able to perform both hydrodynamic and electro-kinetic injection. Separations were done in a fused-silica capillary of 37 cm (30 cm to the detector window)×50 μm I.D.

2.2.1. Method 1 with hydrodynamic injection

An aliquot of 200 μl of the sample (ofloxacin dissolved in HBSS, pH 7.4) was spiked with 50 μl I.S. (procaine hydrochloride 270 μg/ml, dissolved in water). The running buffer solution contained 50 m*M* sodium dihydrogenphosphate and was adjusted to the desired pH with 50 m*M* phosphoric acid. Running buffers with a pH of 2.4, 2.6, 2.8 and 3 were prepared for determining the influence of pH on the migration time of procaine hydrochloride. To 100 ml of the running buffer, 4 g methyl β-CD were added. The detection wavelength was set at 300 nm with a bandwidth of 10 nm. Before use, the capillary was rinsed with 0.1 *M* NaOH for 0.5 min, followed by distilled water for 2 min and then equilibrated for 2 min with the buffer containing the chiral selector. The samples were injected hydrodynamically at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa) for 20 s. Separation was performed at a voltage of 20 kV.

2.2.2. Method 2 with electrokinetic injection

An aliquot of 200 μl of the sample (ofloxacin dissolved in HBSS, pH 7.4) was spiked with 50 μl of I.S. (enrofloxacin 378 ng/ml). Dichloromethane (1 ml) was added to the solution and shaken for 2 min. After centrifugation for 5 min at 2500 rpm, 900 μl of the dichloromethane phase were transferred into a new extraction tube. The organic solvent was evaporated under nitrogen atmosphere. Finally, the residue was dissolved in 200 μl of water containing 10% methanol. The complete process is carried out under light protection. The pH of the phosphate buffer was adjusted to the required pH by addition of phosphoric acid to the given concentration of dihydrogenphosphate solution with both solutions being equimolar. The detection wavelength was set at 280 nm with a bandwidth of 10 nm. Before use, the same conditioning steps were applied for equilibration of the capillary as described in method 1. The samples were injected electrokinetically at 10 kV for 20 s. Separation was performed at 20 kV.

3. Results and discussion

3.1. Methods

3.1.1. Method 1

Method 1 is supposed to serve as a conventional control. The migration time of procaine was strongly dependent on changes of the pH value. Due to the differences in the chemical structure, the two substances show different mobility and therefore different migration times at different pH values. Fig. 2 shows the migration time lag of procaine hydrochlo-
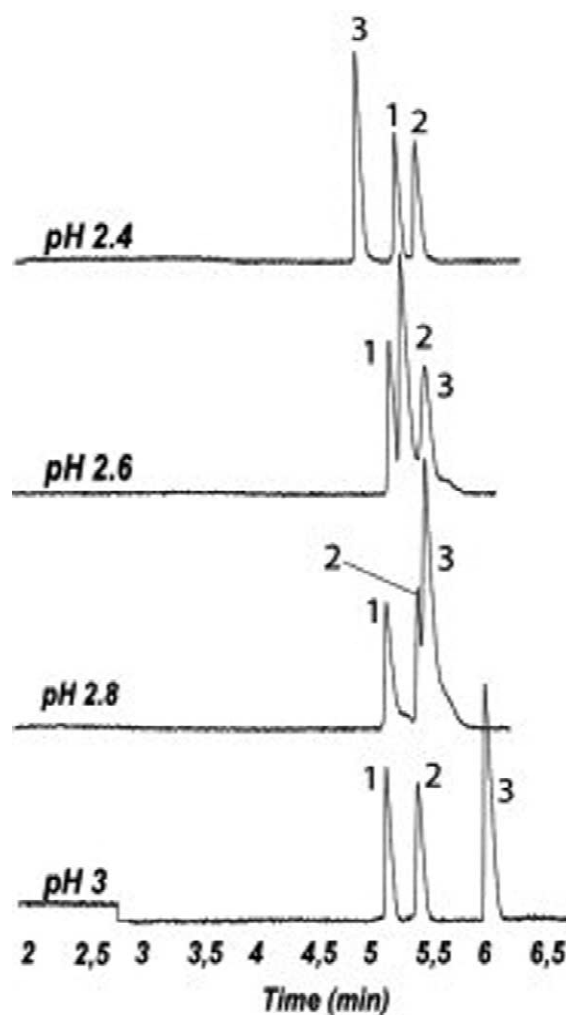
Fig. 2. Electrophoretic separations at different pH values. Separation of ofloxacin enantiomers and the dependence of the migration time of procaine as internal standard on pH change.1 = *S*-ofloxacin; 2 = *R*-ofloxacin; 3 = procaine hydrochloride.

ride in comparison to ofloxacin in response to the pH change. To evaluate the linearity and to determine the limit of detection, eight solutions of racemic ofloxacin were prepared between 18 and 300 μg/ml. Each of these solutions was injected four times. Regression curves were obtained by plotting peak area ratio (ofloxacin enantiomer peak area divided by procaine peak area) versus concentration of this enantiomer, using the least-squares method. The method shows linearity in the concentration range of 9–150 μg/ml for each of the single enantiomers, with a correlation coefficient of 0.9998. This method with hydrodynamic injection mode and without sample preparation led to a limit of detection of 2.5 μg/ml for each enantiomer.

3.1.2. Method 2

It is well known that the sensitivity in CE can be improved by using electrokinetic injection. To increase the amount of sample migrated into the capillary by using the electrokinetic injection, the sample must be free from ions. Fluid–fluid extraction with dichloromethane therefore was carried out. Using the fluid–fluid extraction and electrokinetic injection, the ofloxacin isomers could be measured directly in the lower ng/ml range. Due to mobility differences between procaine hydrochloride and ofloxacin a dramatic drift of the ratio (area ofloxacin:area procaine) was noticed when repeated injections of the sample were made. Therefore an I.S. with similar mobility to ofloxacin was employed [20]. Unlike method 1 no migration time lags appeared at different pH values since enrofloxacin (I.S.) shows a similar mobility to ofloxacin. To optimise this method and to find the optimal conditions for our purpose, a central composite design was carried out.

3.2. Statistical experimental design

To optimise the analytical method a central composite design (135 experiments = 5×27) was chosen as a 2^4 full factorial design with eight face-centered experiments and three supplementary trials at the centre. Four relevant factors were investigated: the background electrolyte concentration, the methyl-β-CD concentration, the buffer pH and the temperature. The values of the experimental factors are summarised in Table 1. The effect of each factor was examined by means of four responses. The four responses examined, in order to obtain the optimal analytical conditions, are the amount of ofloxacin isomers migrated into the capillary, measured by the sum of corrected areas of both enantiomers, the migration time of *S*-ofloxacin as well as the generated current and the resolution between the ofloxacin enantiomers. The selected experiments are shown in Table 2. Finally, it was possible to determine the region in which the optimum values of such variables are simultaneously obtained.

The resolution (R_s) is calculated from the formula according to the European Pharmacopoeia supplement 2001:

$$R_s = \frac{1.18(t_b - t_a)}{b_{0.05a} + b_{0.05b}} \quad t_b > t_a$$

where t_a and t_b are the migration time of the first and second ofloxacin enantiomers, respectively, and $b_{0.05a}$ and $b_{0.05b}$ represents their peak widths measured at half height of the peak.

3.3. Software

Optimised conditions were calculated using UN-

Table 1
Values of experimental factors

Level	X_1, buffer concentration (mM)	X_2, CD concentration (m/V%)	X_3, buffer pH	X_4, temperature (°C)
−1	30	3	2	15
0	50	4	2.5	20
+1	70	5	3	25

Table 2
Central composite design of four factors with selected responses; all experiments ($n = 135$) were randomised

Trial	Experimental factors				Measured responses			
	X_1	X_2	X_3	X_4	Area	R_s	Time (min)	Current (μA)
1	-1	-1	-1	-1	7535	0.81	5.45	34
2	-1	-1	-1	1	7550	0.63	4.32	40
3	-1	-1	1	-1	3541	1.93	5.60	16
4	-1	-1	1	1	5818	1.33	4.38	20
5	-1	0	0	0	5396	1.48	5.27	19
6	-1	1	-1	-1	9496	0.98	5.89	33
7	-1	1	-1	1	6276	1.09	4.65	39
8	-1	1	1	-1	3235	2.24	6.37	16
9	-1	1	1	1	2970	1.98	4.99	19
10	0	-1	0	0	6044	1.85	5.51	31
11	0	0	-1	0	8123	1.50	5.46	42
12	0	0	0	-1	8503	2.00	6.93	27
13	0	0	0	1	8686	1.59	5.33	33
14	0	0	1	0	3671	2.46	6.04	28
15	0	1	0	0	8086	1.95	6.26	29
16	1	-1	-1	-1	8655	1.93	6.62	46
17	1	-1	-1	1	6405	1.68	5.31	54
18	1	-1	1	-1	7038	2.10	6.68	35
19	1	-1	1	1	8204	1.69	5.18	43
20	1	0	0	0	5598	2.37	6.43	39
21	1	1	-1	-1	7967	2.14	7.11	44
22	1	1	-1	1	5395	1.94	5.54	52
23	1	1	1	-1	5798	2.65	7.70	33
24	1	1	1	1	5946	2.23	5.84	41
25	0	0	0	0	5086	2.13	6.08	31
26	0	0	0	0	5908	2.05	6.07	31
27	0	0	0	0	6802	1.88	6.09	30

SCRAMBLER 7.01 (Camo ASA, Norway) and STAT-GRAPHICS plus 5.0 (Manugistics, USA).

3.4. Regression modelling

A second-degree regression for the responses was postulated using the central composite design data:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2$$
$$+ \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{22} X_2^2$$
$$+ \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{33} X_3^2 + \beta_{34} X_3 X_4$$
$$+ \beta_{44} X_4^2$$

where Y represents the experimental response, X_i the independently evaluated factors, B_0 the intercept and β_{ij} the parametric coefficients of the model obtained by multiple regression (first-order interactions).

Table 3 shows the coefficients calculated for each response. Table 4 shows the main effects of the factors, their interactions and the effects of the quadratic parameter in the model with their degree of significance. The statistical data (Table 5) show that both the residual and the experimental standard deviation were of the same order of magnitude, except for the peak area. The high standard deviation of the peak area can be explained by the high deviation of electrokinetic injection. However, the peak area ratio (ofloxacin:enrofloxacin) does not vary even if the conditions are changed. For this reason, corrected peak area of ofloxacin was used instead of peak area ratio in all experiments to study the influence of different conditions on the amount of ofloxacin migrated into the capillary by electrokinetic injection. The coefficients of determination (R^2) and the values of the adjusted coefficients of de-

Table 3
Estimated regression coefficients for selected responses

Coefficients	Peak area	Resolution	Time (min)	Current (μA)
β_0	6503.47	1.988	6.009	30.113
β_1	511.54	0.348	0.527	8.500
β_2	-312.26	0.182	0.296	-0.726
β_3	-1177.66	0.328	0.134	-7.395
β_4	-250.95	-0.146	-0.712	3.243
β_{11}	-1291.99	-0.046	-0.128	-0.886
β_{12}	-170.37	-0.001	0.015	-0.203
β_{13}	866.01	-0.187	-0.013	1.992
β_{14}	-144.78	-0.020	-0.080	0.798
β_{22}	275.96	-0.070	-0.088	0.200
β_{23}	-352.57	0.060	0.099	-0.041
β_{24}	-44.73	0.042	-0.055	-0.144
β_{33}	-892.28	0.009	-0.228	5.330
β_{34}	709.55	-0.074	-0.044	-0.323
β_{44}	1804.97	-0.175	0.158	0.447

termination (R^2_{adj}) were higher than 0.90, except for the peak area, indicating good predictability of the model. The peak area with the lower coefficient of determination showed nevertheless a clear trend when the predicted values were plotted versus the observed values.

Table 4
Effects of the factors and their interactions

Variable	Peak area	Resolution	Time (min)	Current (μA)
Buffer (X_1)	$+++$	$+++$	$+++$	$+++$
M-β-CD (X_2)	$---$	$+++$	$+++$	$---$
pH value (X_3)	$---$	$+++$	$+++$	$---$
Temperature (X_4)	$--$	$---$	$---$	$+++$
X_{11}	$---$	Ns	$--$	$---$
X_1X_2	Ns	Ns	Ns	$-$
X_1X_3	$+++$	$---$	Ns	$+++$
X_1X_4	Ns	Ns	$---$	$+++$
X_{22}	Ns	Ns	Ns	Ns
X_2X_3	$--$	$++$	$+++$	Ns
X_2X_4	$---$	$+$	$---$	Ns
X_{33}	Ns	Ns	$---$	$+++$
X_3X_4	$+++$	$---$	$--$	$---$
X_{44}	$+++$	$---$	$+++$	Ns

$+$ positive effect; $-$, negative effect; Ns, not significant; the degree of significance of the different effects is detailed as below

P value	Negative effect	Positive effect
$\geqslant 0.05$	Ns	Ns
$0.01–0.05$	$-$	$+$
$0.001–0.01$	$--$	$++$
<0.001	$---$	$+++$

Table 5
Statistical data

Variable	Peak area	Resolution	Time (min)	Current (μA)
SD_{exp} [a]	826.441	0.150	0.110	0.623
SD_{res} [b]	1286.570	0.156	0.142	0.747
MAE [c]	922.835	0.115	0.096	0.560
R^2 [d]	63.845	91.386	97.226	99.511
R^2_{adj} [e]	59.627	90.381	96.902	99.454

[a] SD_{exp}, experimental standard deviation ($n = 15$).
[b] SD_{res}, residual standard deviation ($n = 120$).
[c] MAE, mean absolute error-average of the residuals ($n = 120$).
[d] R^2, coefficient of determination.
[e] R^2_{adj}, adjusted coefficient of determination.

3.5. Optimised conditions

A multiple response optimisation was achieved using the desirability method [21]. However, in order to find the best compromise between the four responses, a multicriteria decision making was considered and total desirability function D that weights the responses, together with one single criterion, was used to optimise the four responses simultaneously. To determine the optimal conditions, the point of maximum desirability was calculated by maximizing the peak area and resolution, minimizing the migration time in order to achieve rapid enantioseparation and minimizing the current. The area and resolution, as key responses, were weighted statistically two times more than the migration time and the current in the desirability function. The desirability response surface (Fig. 3) was drawn using STATGRAPHICS as a three-dimensional plot of two factors (buffer concentration and buffer pH) while the chiral selector concentration was kept constant at level 0 and the temperature at level 1.

A typical electropherogram obtained applying the optimised conditions (pH 2.8; chiral selector concentration 4.0%; buffer concentration, 50 mM; temperature, 25 °C) is presented in Fig. 4.

3.6. Method validation

3.6.1. Linearity

Detector response linearity (peak area ratio of ofloxacin:enrofloxacin versus concentration) was evaluated by preparing nine calibration samples
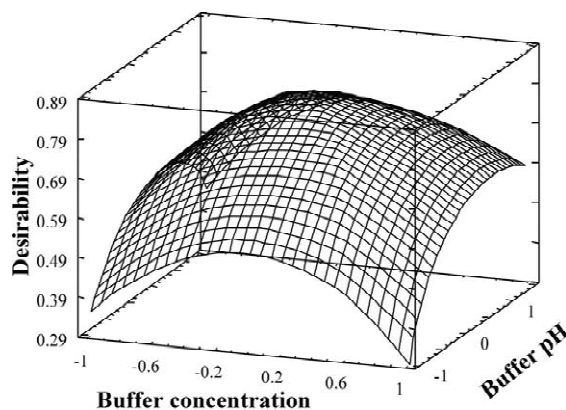

Fig. 3. Desirability response surface. Graphical representation of desirability function (D). Buffer pH is plotted against buffer concentration, maintaining chiral selector concentration at the central level 0 and temperature at level 1. This desirability response surface is drawn using STATGRAPHICS 5.0.

using racemic ofloxacin in the concentration range of 40–1400 ng/ml. Regression curves were obtained by plotting peak area ratios (ofloxacin peak area divided by enrofloxacin area as I.S.) versus concentration, using the least-squares method. The determination coefficients R^2 were >0.999 for both enantiomers. The limit of detection (LOD) represents the smallest peak which can be quantified with accuracy. The
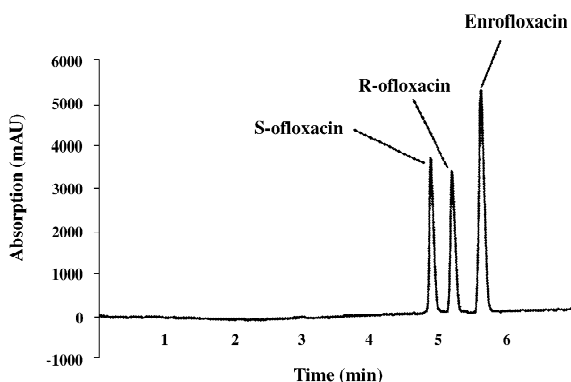

Fig. 4. Typical electropherogram using the optimised conditions. Separation of enantiomers of ofloxacin under the optimised conditions: 50 mM buffer phosphate, pH 2.8, containing 4% chiral selector; fused-silica capillary 37 cm (30 cm effective length)×50 μm I.D.; applied voltage 20 kV, detection at 280 nm; temperature 25 °C; injection time 20 s at 10 kV.

LOD was determined as three times the signal-to-noise ratio and the limit of quantitation (LOQ) as nine times the signal-to-noise ratio. The estimated LOD, LOQ and the regression data obtained are the same for both enantiomers (Table 6).

3.6.2. Precision

The repeatability and intermediate precision of migration times and peak area ratio were determined. The repeatability (within-day precision) of the method and the intermediate precision were determined by performing replicate injections ($n=16$) of 600 ng/ml solution containing racemic ofloxacin and the internal standard. The standard deviation (RSD) values for migration time and peak area ratio of ofloxacin enantiomers are shown in Table 7. The RSD values of the repeatability were in all cases <0.8% for the peak area and <0.4% for the migration time. Intermediate precision was also evaluated over a 3-day period by performing four injections daily.

3.6.3. Recovery rate after extraction

In order to study the recovery rate of each enantiomer of ofloxacin from the HBSS, 1 ml of blank HBSS spiked with known amounts of racemic ofloxacin was extracted as described in Section 2.2.2. The I.S. was added after the extraction procedure. The recovery rate of the extraction was more than 92% for each enantiomer of ofloxacin.

3.7. Application

The developed method has been used for preliminary investigations of the absorption of ofloxacin

Table 7
Method precision given as RSD (%) values

	S-Ofloxacin	*R*-Ofloxacin
Repeatability		
Migration time	0.32	0.29
Peak area ratio	0.58	0.79
Intermediate precision		
Migration time	1.56	1.63
Peak area ratio	1.36	1.44

enantiomers in vitro (Caco-2 model). The cells were cultured according to previously published procedures [22]. Transport experiments were performed at 37 °C in HBSS using a Transwell filter cluster [23]. A 500 µl volume of the prewarmed 10 m*M* ofloxacin solution in buffered HBSS was added to the apical side. Samples (200 µl) were taken every 20 min from the basolateral side and were replaced with equal volumes of fresh buffered HBSS immediately. The samples were prepared as described in Section 2.2.2 and analysed at the optimal conditions. Fig. 5 shows the amount of *S*- and *R*-ofloxacin transported across Caco-2 cell monolayer.

4. Conclusion

In CE, UV detection is performed on-line and therefore the optical pathlength is dictated by the capillary I.D. This results in a low amount of absorbing drug in the optical path. To overcome this disadvantages of the UV detection in CE, the electrokinetic injection mode can be used as an alternative to the hydrodynamic injection. To use this technique, the samples must be free from ions. In addition, the

Table 6
Regression data for the calibration curves

	S-Ofloxacin	*R*-Ofloxacin
Range (ng/ml)	20–700	20–700
Regression equation ($n=9$)	$y = -0.0102 + 0.0016x$	$y = -0.0071 + 0.0016x$
Determination coefficient (r^2)	0.9993	0.9993
LOD (ng/ml)	3.8	3.6
LOQ (ng/ml)	11.4	10.8

Calibration data of ofloxacin enantiomers at the optimal conditions, achieved by the experimental design (central composite design). Peak area ratio versus sample concentration (ng/ml). Instrument: Beckman P/ACE 5500; experimental conditions: fused-silica capillary 37 cm×50 µm I.D., electrophoretic injection for 20 s at 10 kV; temperature 25 °C; voltage at 20 kV; UV detection at 280 nm; electrolyte system, 50 m*M* sodium phosphate buffer, pH 2.8 containing 4.0% methyl β-cyclodextrin.
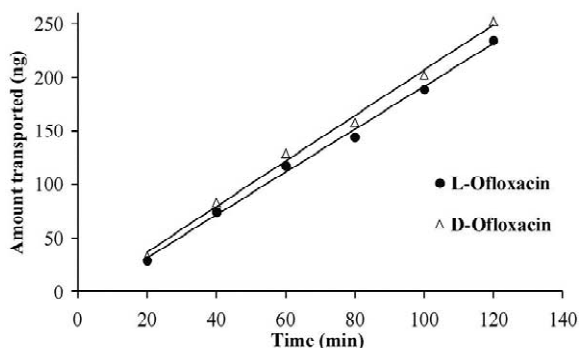
Fig. 5. Determination of the amount of absorbed L- and D-ofloxacin using Caco-2 cells. Amount transported versus time of L- and D-ofloxacin across Caco-2 cell monolayers (transport direction: apical to basolateral). The donor concentration was 10 μM racemic ofloxacin.

mobility of the chosen internal standard must be similar to that of the analyte to avoid migration time lags and receive a constant area ratio, when repeated injections of the sample were made.

The use of experimental design strategies for optimisation and robustness testing allowed a development of an analytical method to quantify the ofloxacin enantiomers in the ppb-range with methyl β-CD as chiral selector. The possibility of dealing with several responses at a time, obtaining the best compromise between different goals, was a very important aspect, since separative techniques usually require a simultaneous optimisation of several responses. In our method, increasing the buffer concentration improves the resolution and increases the amount of the analyte migrating into the capillary; however, it produces unwanted effects on the migration time and current. While the pH has a positive effect on the resolution, it increases the migration time. The chiral selector concentration shows similar effects on all responses like the pH. We are able to show, that the amount of analyte migrated into the capillary by using the electrokinetic injection mode, can be increased significantly by optimisation of the conditions, improving sensitivity. The optimised method proved to be suitable for the quantitative analysis of ofloxacin enantiomers in high diluted samples for in vitro studies using the Caco-2 model.

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