Theo de Boer
Roelof Mol
Rokus A. de Zeeuw
Gerhardus J. de Jong
Kees Ensing

Department of Analytical Chemistry and Toxicology, University Center for Pharmacy, Groningen, The Netherlands

Enantioseparation of ofloxacin in urine by capillary electrokinetic chromatography using charged cyclodextrins as chiral selectors and assessment of enantioconversion

A method was developed for the enantioseparation of ofloxacin, a member of the fluoroquinolones, using an anionic cyclodextrin-derivative with or without combination with a neutral cyclodextrin-derivative, as the chiral selector (s) in an electrokinetic chromatography system. The best results were obtained with 0.35 mM sulfated β-cyclodextrin dissolved in a 50 mM phosphate buffer, pH 2.5, and at 15°C. Under these conditions, a resolution of 2 was readily achieved. Furthermore, under adequate separation conditions, studies were performed in order to assess possible *in vitro* and *in vivo* enantioconversion of levofloxacin. The current method allows detection of 2 μg *R*-(+)-ofloxacine/mL diluted urine without the necessity of sample cleanup.

Keywords: Anionic cyclodextrins / Capillary electrophoresis / Chiral selectors Ofloxacin / Selectivity EL 4308

1 Introduction

Ofloxacin (Fig. 1) is a member of the fluoroquinolones, a class of synthetic antimicrobial agents. The fluoroquinolones are recently gaining much interest because they provide activity against both Gram-positive (fluor atom) and Gram-negative (piperazine group) organisms, bearing little side effects [l,2]. Ofloxacin is used for the treatment of (both uncomplicated and complicated) urinary tract infections, gonorrhea, nongonococcal urethritis, and cervitis [3]. Pharmacological research has shown that the antibacterial activity of the *levo*-enantiomer of ofloxacin (*S*-(–)-ofloxacin or levofloxacin) is 8–128 times higher than that of *R*-(+)-ofloxacin and approximately two times higher than that of the racemate [4]. It should be noted that levofloxacin is excreted for approximately 93% unchanged in the urine within 24 h, whereas the urinary excretion of the unchanged racemate is approximately 75%, within 24 h. For both the racemate and levofloxacin it has been found that only 3% is metabolized [3]. Nowadays, the US Food and Drug Administration (FDA), as well as regulatory authorities in Europe, China, and Japan, have provided guidelines indicating that preferably only the active enantiomer (eutomer) of a chiral drug should be brought to the market [5–9]. For that reason, and in general to monitor the enantiomeric purity of the eutomer, it is mandatory to develop enantioselective separation methods for every enantiomeric drug that has been or will be brought to the market.

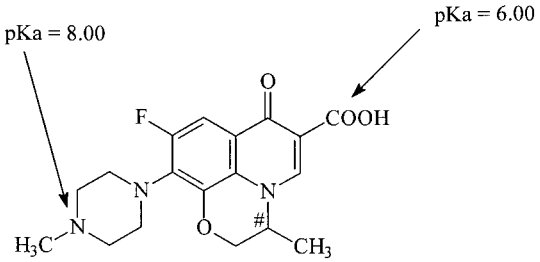

Figure 1. The molecular structure of ofloxacin with the approximate pK_a values [38]. The p*I* value is 6.97 [39]; # indicates the chiral center.

Zeng *et al.* [10] recently described a method for the direct chiral Separation, *i.e.* without the necessity of derivatization, of ofloxacin enantiomers in microsomal incubates by ligand-exchange reversed phase-liquid chromatography (RP-LC) with fluorescence detection. They were able to monitor the stereoselective metabolism of ofloxacin in the glucuronidation process by obtaining baseline separations (resolutions between 1.69–1.75) of the ofloxacin enantiomers within 25 min. However, it should be noted that for impurity profiling and the monitoring of possible *in vivo* or *in vitro* enantioconversion a high resolution is required to quantitate both enantiomers, especially when the distomer (smaller peak) is located at the tail of the eutomer (larger peak) [11, 12]. To achieve resolutions exceeding 2.0 in LC using the method described by Zeng

Correspondence: Theo de Boer, Department of Analytical Chemistry and Toxicology, University Center for Pharmacy, A. Deusinglaan 1, Postbus 196, 9700 AD Groningen, The Netherlands
E-mail: t.de.boer@farm.rug.nl
Fax: +31-50-3637582

Abbreviations: D.S., degree of substitution; **HP-β-CD**, hydroxypropyl-β-cyclodexttrin; **S-β-CD**, sulfated β-cyclodextrin

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et al., unacceptably long retention times would be necessary. Therefore, for rapid and efficient chiral separations, the use of capillary electrophoresis (CE) seems a useful alternative. Some major advantages of chiral separations in CE in comparison with LC are the low consumption of the chiral selector and the high plate numbers due to a reduced peak broadening as a consequence of the absence of Eddy diffusion and mass transfer between two phases (the *A*- and *C*-term of the Van Deemter equation [13], respectively). This implies that because of the high plate numbers combined with a high selectivity, baseline separations can be achieved more readily. On the other hand LC, in comparison to CE, offers a higher concentration sensitivity and is capable to detect impurities at a lower level.

In the literature, only a few chiral separations of ofloxacin using CE have been mentioned. However, none of these papers describes the chiral separation of ofloxacin in a biological matrix. Exotic chiral selectors like vancomycin, and bovine or human serum albumin (BSA/HSA) were described by several authors [14–18]. Horimai *et al.* [19] recently described the use of an γ-CD-Zn(II)-D-phenylalanine complex to separate several new quinolone drugs. Fu *et al.* [20] used a more straightforward and well-described approach [21–25] for the chiral separation of ofloxacin, namely the use of neutral derivatized cyclodextrins (CDs) as chiral selectors in EKC Systems. The use of CDs as chiral selectors in CE systems was first described by Fanali in 1989 [26] and is considered to be a EKC mode, because the change in migration rate of the enantiomers is a direct consequence of the interaction (formation constants) of the analyte with a pseudostationary phase [27]. A relatively new approach is the use of charged CDs because of their ability to perform fast chiral separations at low concentrations and because of the potential for chiral separation of neutral racemates [28]. Three types of charged CDs have been used in chiral capillary electrophoretic separations (anionic, cationic, and amphoteric CDs) [25, 29–34], sometimes in combination with neutral CDs [24, 29, 31, 36]. The principles and applications of these so-called dual CD Systems have been described in the literature and may provide the opportunity to fine-tune the separation system. The neutral CDs may stereoselectively influence the free fraction of the chiral analyte available for complexation with the charged CDs, with the possibility to change the migration order of the enantiomers and without increasing the electrical current over the capillary [36].

This paper describes the use of a commercially available anionic β-CD, sulfated β-CD (S-β-CD degree of sustitution, (D.S.) = 7–11) in combination with neutral (substituted) α-,β-, and γ-CD as chiral selectors in a dual CD-system for the enantioseparation of ofloxacin. Furthermore, under adequate separation conditions, studies were performed in order to assess possible *in vitro* and *in vivo* enantioconversion of levofloxacin. Levofloxacin was prescribed to hemophilict patients who were suffering from infections due to an immune-deficiency manifested by the administration of cytostatics. The possible *in vitro* enantioconversion (chemical enantioconversion) was studied by adding levofloxacin to a diluted human urine and to a 10^{-5} M HCl solution. Possible enantioconversion was monitored over two weeks by injection of the testsolutions into the chiral CE system. The *in vivo* enantioconversion (metabolic or chemical) of levofloxacin was investigated in urine samples from 20 subjects.

2 Materials and methods

2.1 Instrumentation

Analyses were carried out on a Beckman P/ACE System 5500 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector. The bare fused-silica capillary with an outer polyimide coating (50 μm ID., 375 μm OD) was from Composite Metal Services (Hallow, UK). Data acquisition was performed with P/ACE 5000 Series Software. The vials used were 4 mL glass vials sometimes with a 0.7 mL glass insert obtained from Phase Sep (Waddinxveen, The Netherlands)

2.2 Chemicals and solutions

The CE runbuffers were prepared by mixing 50 mM *ortho*-phosphoric acid (Merck, Darmstadt, Germany) with 50 mM sodium dihydrogen phosphate monohydrate (Merck) until the desired pH was obtained (pH 2.5). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). The conductivity of the purified water was at a constant 18.2 MΩ. Levofloxacin (*S*-(–)-ofloxacin) *hemi*-hydrate (TAVANIC®) was from Hoechst Marion Roussel (Frankfurt am Main, Germany) and the hydrochloric acid salt of racemic ofloxacin was obtained as an Infusion liquid with a concentration of 2 mg/mL in an isotonic hydrochloric acid solution, pH 5.0 (TARIVID®; Hoechst). Both were a gift from the Academic Hospital of the Free University in Amsterdam (The Netherlands). Solutions of racemic ofloxacin were prepared by diluting the infusion liquid with runbuffer to a final concentration of 20 μg *R*(+), *S*-(–)-ofloxacin per mL. Stock solutions (1 mg/mL of levofloxacin were prepared in methanol (Merck) which were diluted just before use with runbuffer to a final concentration of 10 μg/mL. The patient-urine samples were

Table 1. Effect of HP-β-CD and S-β-CD at different concentrations on resolution of racemic ofloxacin

HP-β-CD			S-β-CD		
Conc. (mM)	α	*Rs*	Conc. (mM)	α	*Rs*
0	–	–	0	–	–
1	–	–	0.1	1.012	< 1.5
2	1.014	< 1.5	0.15	1.016	< 1.5
5	1.030	1.5	0.20	1.027	< 1.5
10	1.045	2.0	0.25	1.034	1.5
15	1.039	2.0	0.40	1.064	2.7
			0.60	1.105	3.5
			0.80	1.146	4.3
			1.0	1.151	4.3

Injections at 3 s, 0.5 psi and separation at 25 kV

diluted 10 times with water, to exclude irregular peak shapes. The *in vitro* enantioconversion of levofloxacin was studied in diluted human urine. For these studies drug free urine from volunteers was diluted 10 times with water, whereafter levofloxacin was added to a final concentration of 10 μg/mL diluted urine. The same concentration was used for studies in an aqueous solution brought to pH 5 with hydrochloric acid in order to mimic the actual ofloxacin formulation. Both solutions were kept for two weeks in reduced light at 37°C (to quicken the process), during which daily samples were taken and analyzed. The *in vivo* enantioconversion of levofloxacin was studied in patient urine. For these studies the urine samples were diluted 10 times with water, containing approximately 10 μg levofloxacin per 1 mL of diluted urine. The patient urine samples were gathered from hemophilic patients (n = 20), who were administered 500 mg levofloxacin daily during a period of at least one month. Within 10 h after the first administration, urine samples were taken and stored in a freezer at –80°C for a period of at least 14 days (with a maximum of 36 days). The patient-urine samples were defrosted to room temperature just before use and analyzed using the developed chiral separation system. Highly sulfated β-CD (D.S. = 7–11) and β-CD were obtained from Sigma (Zwijndrecht, The Netherlands). Hydroxypropyl-β-CD (HP-β-CD, D.S. = 1.0) was obtained from Wacker-Chemie (Munich, Germany). α-CD was a gift from AVEBE (Foxhol, The Netherlands) and γ-CD was a gift from the Department of Pharmaceutical Technology of the University of Groningen, The Netherlands. All solutions were filtered through disposable syringe sample-filters obtained from Upchurch Scientific (Oak Harbor, WA, USA) and degassed for 5 min in an ultralsonic bath (50 kHz; Branson Europa B. V., Soest, The Netherlands), immediately prior to use.

2.3 CE conditions

A capillary with a total length of 37 cm and an effective length of 30 cm was used. An optical window with a length of 2 mm, obtained by burning off the polyimide coating, was aligned with the UV detection cell. New capillaries were rinsed for 10 min at 20 psi (1313 mbar) with 1 M sodium hydroxide, water, 1 M hydrochloric acid, water and with the run buffer, respectively. Maximum absorption for the drugs was observed at a wavelength of 291 nm and the diode array detector was consequently set to monitor the effluent at 291 nm. At this particular wavelength there is no or negligible interference of the matrix compounds. CDs were dissolved in the runbuffer and hydrodynamically injected until the capillary was fully filled. The capillary was mounted in a cartridge and thermostated at 15°C. Injections were done at 0.5 psi (33 mbar) for different periods and separation took place at 25 kV. The ramp time of the applied voltage was 0.17 min.

3 Results and discussion

3.1 Enantiomeric separation of ofloxacin

As can be deducted from Fig. 1, ofloxacin is an amphoteric substance with a p*I*value of 6.97. At a pH of 2.5 the substance is fully protonated. CE separations were performed using either HP-β-CD, α-CD, β-CD, γ-CD, or sulfated-β-CD at different concentrations. Only the hydroxypropyl-substituted CD and the sulfated CD were able to resolve the enantiomers within 7 min ($Rs > 1.5$). In Table 1, the selectivity factors (defined as: $\alpha = t_2/t_i$, where t = migration time for the first (1) and the last (2) eluting enantiomer) and the resolutions (defined as: $Rs = 2\,\Delta t/W_1+W_2$, where Δt_r is the difference in migration time of the two analytes and W is the peak width on the baseline for the first (1) and the last (2) eluting peak) are presented. As was anticipated, baseline separation of the ofloxacin enantiomers with the sulfated CDs could be readily achieved at much lower concentrations than with the hydroxypropyl-substituted CDs. Furthermore, the maximum obtained resolution is at least a factor 2 higher with the charged CD than with the neutral CD. Concentrations higher than 1.0 mM could not be used, due to severe baseline fluctuations. A baseline separation, *i.e.*, $Rs > 1.5$, of ofloxacin in 10× diluted (drug-free) human urine could be obtained by using a 0.2 mM S-β-CD solution, within 4 min as shown in Fig 2. The electropherograms also show the good selectivity which can be obtained for the direct injection of 1:10 diluted urine.

A critical note on using negatively charged CDs in a normal polarity CE system should, however, be given. Because the mobility of the negatively charged CD is towards the anode, it should be warranted that the net

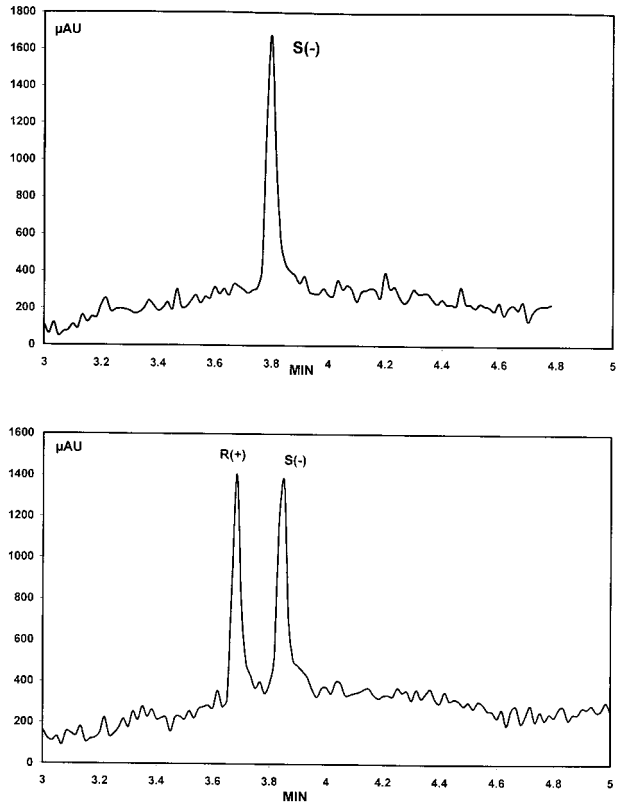

Figure 2. Electropherograms of racemic ofloxacin in (a) 5 mM phosphate buffer, pH 2.5 and (b) 0.25 mM S-β-CD, added to the same buffer. Sample, drug-free human urine obtained from a volunteer (10 × diluted with water) spiked with 20 µg ofloxacin per mL diluted (1:10) urine. Sample injection: 3 s at 0.5 psi; EK separation at 15°C (25 kV); UV-detection at 291 nm. *R*(+) = *R*-(+)-ofloxacin; *R*(–) = *R*-(–)-ofloxacin or levofloxacin.

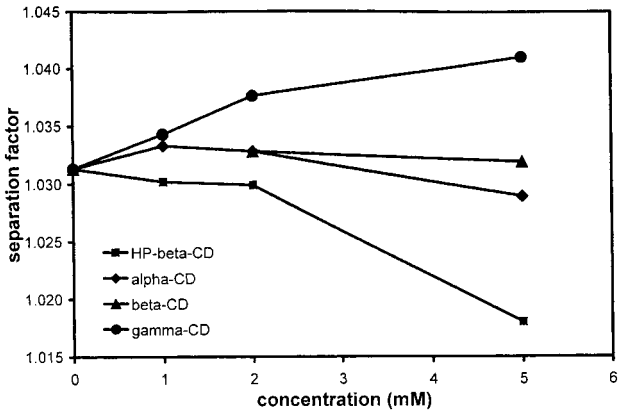

Figure 3. The selectivity factors (α) obtained by dual CD Systems. The S-β-CD concentration was always 0.25 mM.

mobility of the analyte/S-β-CD complex is towards the cathode for the obvious reason that otherwise the peaks will not be detected. The latter is dependent on the S-β-CD concentration. When the desired resolution is not obtained before the critical S-β-CD concentration is reached or when electrodispersion is observed, dual CD-systems, *i.e.*, combinations of a neutral CD and S-β-CD may be an alternative. Therefore, the next step was to investigate the tuning effect of several neutral CDs added to an anionic CD solution on resolution. For that reason we added either α-CD, HP-β-CD, β-CD or γ-CD in various concentrations (0–5 mM to a 0.25 mM S-β-CD solution (stock solution). This S-β-CD concentration (0.25 mM) was the minimal concentration that was able to achieve baseline separation. For that reason, it was chosen to observe the tuning effect of the addition of other neutral chiral selectors on resolution and selectivity factors.

In Fig. 3, the selectivity factors (α) obtained by using these dual CD Systems are presented. The figure shows the positive effect when γ-CD was added to the stock solution and the negative effect when either HP-β-CD or α-CD was added to the system. It can be concluded that although it is possible to fine-tune the selectivity of the separation system, relatively large amounts of neutral CD-derivatives are necessary and, moreover, only a small increase of the S-β-CD concentration could result in the same increase of the selectivity. The most interesting feature is probably the effect of HP-β-CD on both separation factor and resolution. As can be seen from Table 1, this neutral substituted CD is capable of baseline separation of ofloxacin. However, the combination of HP-β-CD with S-β-CD as chiral selector in the same run seems to eliminate the ability of chiral recognition of the individual CDs derivatives. The "quenching" effect of this particular dual CD system could be explained if there was a difference in migration order of the enantiomers as a consequence of complexation with the CD-derivatives. However, the use of either CD-derivative leads to the same enantiomer mobility with the result that we are not able to explain the observation. Moreover, this is especially interesting while the obtained results contradict with some recently proposed mathematical models [36], which are supposed to be able to predict selectivity in dual CD systems. From these models it was concluded, as was experimentally shown [36, 37], that when both CD derivatives interact selectively with the analyte enantiomers and lead to independent complexation, the enantioseparation will be significantly increased if the affinity pattem and the effect of the two CDs on the analyte mobility are the same.

3.2 Enantioconversion studies of levofloxacin

As was described in Section 3.1, chiral separation of racemic ofloxacin can readily be performed with just a single oppositely charged CD, *i.e.*, without the necessity to use a dual CD system. It is acknowledged that although

a dual CD system could fine-tune the separation, the concentrations of the anionic CD used do not lead to any analysis problems as was addressed in Section 3.1. In order to achieve a resolution of at least 2.0, a 0.35 mM S-β-CD solution was used to monitor the possible *in vivo* and *in vitro* enantioconversion. The hydrochloric acid solution (10^{-5} M) showed no signs of enantioconversion and no other peaks were detected. The spiked diluted urine samples also showed no change in the peak height of levofloxacin, measured at $\lambda = 291$ nm. In all cases, no *in vitro* enantioconversion, detected at $\lambda = 291$ nm, was observed.

To assess the extent of enantioconversion as percentage of the active enantiomer, the detection limit of the formed enantiomer *R*-(+)-ofloxacine forms a limiting factor. Furthermore, the detection might be affected by the presence of a large excess of the active *S*-(–)-enantiomer. On the basis of available pharmacokinetic data, the concentration of the latter enantiomer in urine, collected during the first 10 h after administration, will be about 100 μg/mL. For that reason the detectability of the *R*-(+) enantiomer (2,4,8 and 12 μg/mL) was studied in the presence of 100 μg/mL of the *S*-(–)-enantiomer using 0.35 mM S-β-CD solution as chiral selector.

The experiments show (Fig. 4) that the limit of detection for *R*-(+)-ofloxacine is 2 μg/mL. However, since urine samples have to be diluted 10-fold, as a consequence the detection limit in undiluted urine is 20 μg/mL for *R*-(+)-ofloxacine. Thus, *in vivo* enantioconversion can only be seen when *R*-(+)-ofloxacine exceeds 20%. In all patient urines measured at $\lambda = 291$ nm, the *R*-(+)-enantiomer was not detectable, which illustrates that there is no enantioconversion of the *S*-(–)-enantiomer which exceeds 20%. In order to illustrate the linearity of the detector response, linear regression analysis ($Y = a+bX$) was performed over the range of 2–12 μg *R*(+)-ofloxacin/mL (expressed as the peak height). The following equation was obtained: $Y = -0.1\ (\pm 0.2)+0.72\ X\ (\pm 0.03)$, corresponding with a linearity, expressed as the coefficient of determination of $r^2 = 0.9975$ ($p = 0.00127$; $n = 4$).

4 Concluding remarks

The use of charged CDs for the chiral separation of oppositely charged ofloxacin appears to be an effective method. Combinations of the charged CD with a neutral CD may be useful for tuning the separation system, but can also lead to a severe decrease in resolution. To monitor possible *in vitro* and/or *in vivo* enantioconversion of levofloxacin a concentration of 0.35 mM S-β-CD in a 50 mM phosphate buffer at pH 2.5 was sufficient for baseline separation and resulted in a resolution higher than 2. Under these conditions chiral, separations were obtained within 5 min. The analysis of ofloxacin in urine at a wave-

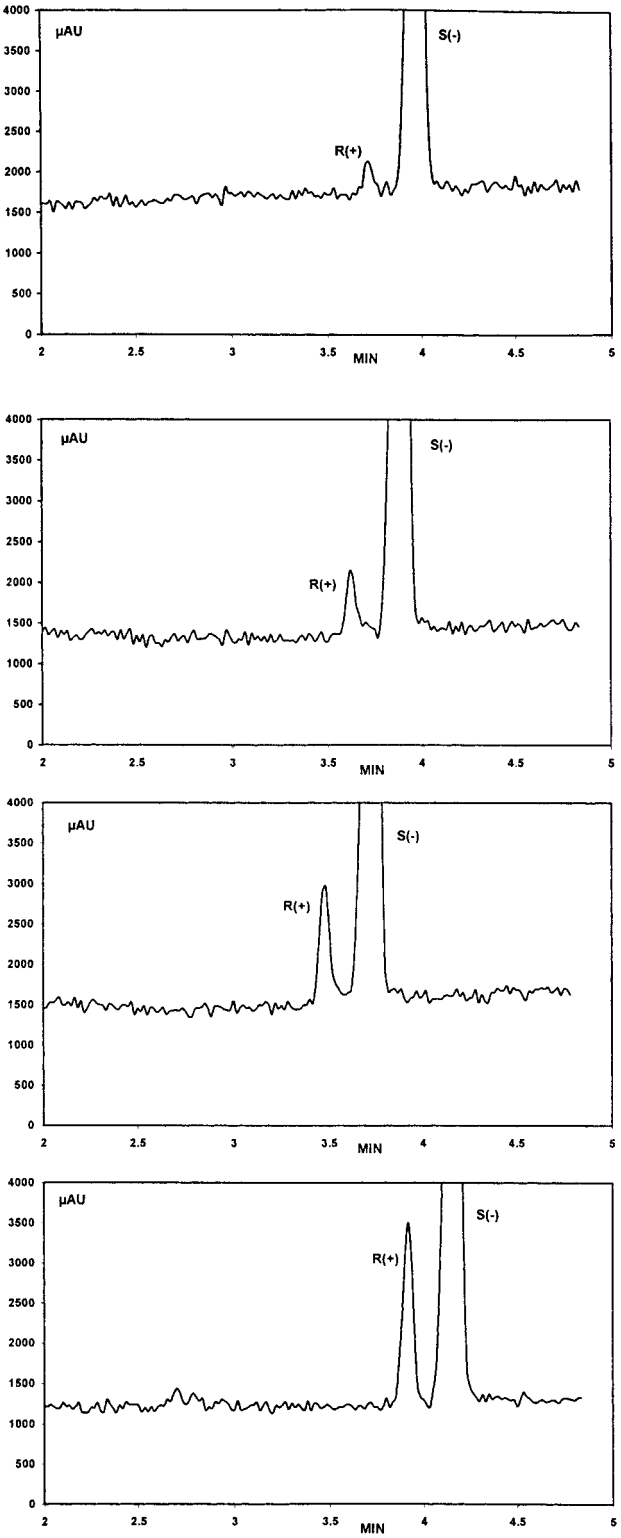

Figure 4. Electropherograms of 2, 4, 8 and 12 μg/mL *R*(+)-ofloxacin and 100 μg/mL *S*(–)-ofloxacin using 0.35 mM S-β-CD as the chiral selector in a 50 mM phosphate buffer, pH 2.5 Sample injection; 3 s at 0.5 psi; EK separation at 15°C (25 kV); UV-detection at 291 nm. *R*(+) = *R*-(+)-ofloxacin; *R*(–) = *R*-(–)-ofloxacin or levofloxacin.

length of 291 nm seems to be very effective with little or no interference of matrixcompounds. Levofloxacin is rather stable in urine, which implies that analysis of the samples can take place even after some time. Furthermore, none of the analyzed urinesamples show demonstrable enantioconversion.

For profiling of drug impurities, degradation products, synthetic precursors, side products, or metabolites in pharmaceuticals in general there is a limit of 0.1%. In the case of enantioconversion in the human body as a consequence of metabolic or chemical conversion, no clear guidelines are available. The current method allows detection of 2 μg *R*-(+)-ofloxacine/mL diluted urine or buffer solution. This amount can be readily detected in the presence of a 50-fold excess of the active enantiomer being 100 μg *S*-(–)-ofloxacine. Yet, it should be noted that the extent of enantioconversion that can be readily detected, is determined by the limit of detection for *R*-(+)-ofloxacine and the actual concentration of S-(–)-ofloxacine present in the diluted urine samples. In an undiluted urine sample the detection limit is consequently 20 μg *R*-(+)-ofloxacine, *i.e.*, approximately 20%. Chemometrical optimization of the separation system, investigating parameters like urine dilution factor and injection volume, could decrease the limit of detection of *R*-(+)-ofloxacine in urine samples. Furthermore the detectability can be improved by extracting the enantiomers from urine and analysis of the concentrated extract, but then the analysis time would be increased.

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