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Stereoselective determination of ofloxacin and its metabolites in human urine by capillary electrophoresis using laser-induced fluorescence detection

Christian Horstkötter, Gottfried Blaschke*

Institute of Pharmaceutical Chemistry, University of Münster, Hittorfstrasse 58–62, D-48149 Münster, Germany

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Dedicated to Prof. Richard Neidlein, Heidelberg, on the occasion of his 70th birthday

Abstract

A capillary electrophoresis method for the simultaneous separation and enantioseparation of the antibacterial drug ofloxacin and its metabolites desmethyl ofloxacin and ofloxacin *N*-oxide in human urine has been developed and validated. Enantioseparation was achieved by adding sulfobutyl β-cyclodextrin to the running buffer. The detection of the analytes was performed by laser-induced fluorescence (LIF) detection using a HeCd-laser with an excitation wavelength of 325 nm. In comparison with conventional UV detection, LIF detection provides higher sensitivity and selectivity. The separation can be performed after direct injection of urine into the capillary without any sample preparation, because no matrix compounds interfere with the assay. Additionally, the high sensitivity of this method allows the quantification of the very low concentrations of enantiomers of both metabolites. The limit of quantification was 250 ng/ml for ofloxacin enantiomers and 100 ng/ml for each metabolites' enantiomers. This method was applied to the analysis of human urine samples collected from a volunteer after oral administration of 200 mg of (±)-ofloxacin to elucidate stereoselective differences in the formation and excretion of the metabolites. It could be demonstrated that the renal excretion of the *S*-configured metabolites, especially *S*-desmethyl ofloxacin, within the first 20 h after dosage, is significantly lower than that of the *R*-enantiomers.

Keywords: Ofloxacin; Desmethyl ofloxacin; Ofloxacin *N*-oxide

1. Introduction

Recently, capillary electrophoresis (CE) combined with laser-induced fluorescence detection has become more and more popular in the analysis of drugs and drug metabolites in biological body fluids [1–3]. Besides such advantages of CE as short analysis times, very small sample volumes (in the range of few nanolitres) and, in comparison with HPLC, the very low consumption of organic solvents, this technique offers the possibility of a simple enantioselective assay when an appropriate chiral selector is added to the running buffer. The extremely high sensitivity of laser-induced fluorescence (LIF) de-

*Corresponding author. Tel.: +49-251-83-33311; fax: +49-251-83-32144.

E-mail address: blaschg@uni-muenster.de (G. Blaschke).

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tection allows the determination of analyte concentrations below 1 μg/ml without any preconcentration procedures. Using a commercially available helium–cadmium laser with a fixed wavelength of 325 nm, this technique can be applied to all compounds that can be excited to native fluorescence around this wavelength.

Ofloxacin (OFLX; Fig. 1) is a fluorinated quinolone antibacterial showing bactericidal effects by inhibition of DNA gyrase [4]. Due to the substitution by a methyl group at the C-3 position of the oxazine ring, the drug is chiral. Although it is well known that the *S*-(−)-enantiomer is 8–128 times more potent than the *R*-(+)-enantiomer and about twice as potent as (±)-ofloxacin [5,6], the drug is still used therapeutically in its racemate form and, since 1998, additionally as the enantiopure *S*-(−)-isomer.

Two metabolites of ofloxacin, desmethyl ofloxacin (DMOF) and ofloxacin *N*-oxide (OFNO; Fig. 1),

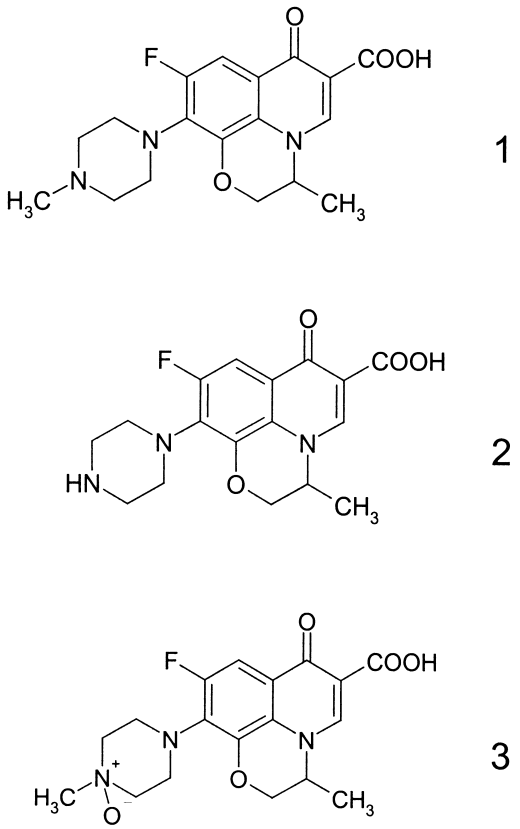

Fig. 1. Chemical structures of ofloxacin (1), desmethyl ofloxacin (2) and ofloxacin *N*-oxide (3).

have been identified at low concentrations in human urine [7,8]. A third metabolite, ofloxacin β-D-glucuronide, is eliminated by biliary excretion in humans and could not be found in urine until recently [9]. Overall, ofloxacin undergoes only limited metabolism: nearly 80% of an orally administered dose is excreted unchanged in urine and less than 5% in the form of metabolites.

The enantioselective determination of ofloxacin in biological body fluids has already been achieved by HPLC after the addition of chiral selectors to the mobile phase [10], using a chiral stationary phase, or by derivatisation of the analyte with a chiral reagent [11]. Applying the last method to a pharmacokinetic study, stereoselective differences in the excretion of *S*-(−)- and *R*-(+)-ofloxacin have already been elucidated [12,13]. The disadvantages of these methods however are the complex sample preparation procedures involved, including extraction and/or derivatisation steps, and the fact that only the enantiomers of the unchanged drug, and not those of the metabolites, were determined.

CE has been used previously to separate the enantiomers of ofloxacin in different experiments using various cyclodextrins [14–18], vancomycin [19], bovine serum albumin [20] or a combination of γ-cyclodextrin, D-phenylalanine and zinc(II) sulfate [21] as chiral selectors, but none of these methods has been validated for quantitative assays or applied to biological samples. In addition, resolution factors were often poor.

In this paper, we present a fully validated CE–LIF method for the simultaneous determination of the enantiomers of ofloxacin and its metabolites in human urine using sulfobutyl β-cyclodextrin as the chiral selector. Sample preparation was not necessary, since the urine could be injected directly into the capillary. Applying this method to the urine samples of a human volunteer, stereoselective differences in the formation and excretion of the metabolites could be demonstrated.

2. Experimental

2.1. Chemicals and reagents

Ofloxacin [(±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-de]-

1,4-benzoxazine-6-carboxylic acid], desmethyl ofloxacin [(±)-9-fluoro-2,3-dihydro-3-methyl-10-(1-piperazinyl)-7-oxo-7*H*-pyrido[1, 2, 3-de]-1,4-benzoxazine-6-carboxylic acid] and ofloxacin *N*-oxide [(±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-4-oxido-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid] were obtained as analytical reference standards from Daiichi Pharmaceutical (Tokyo, Japan). The internal standard (I.S.) quinine sulfate was supplied by Caesar and Loretz (Hilden, Germany).

Sulfobutyl β-cyclodextrin, sodium salt (Advasep® 7; Lot No. ADV7-012-01; molecular formula: $C_{42}H_{70-n}O_{35}(C_4H_8SO_3Na)_n$, $n=6.5$ (average degree of substitution); average molecular mass: 2163 g/mol) was purchased from CyDex Inc. (Overland Park, Kansas, USA).

Orthophosphoric acid (85%), triethylamine and methanol were obtained from J.T. Baker (Deventer, The Netherlands) and sodium dihydrogen phosphate monohydrate from E. Merck (Darmstadt, Germany). Sodium hydroxide solution (0.1 *M*) was purchased from Waldeck (Münster, Germany). Water was purified by bidistillation. Nitrogen was supplied by Messer Griesheim (Krefeld, Germany). All reagents were of analytical grade unless otherwise specified.

Blank urine was obtained from a fasted healthy volunteer and stored at −18°C in a freezer until use. Ofloxacin tablets (Tarivid® 200) and levofloxacin tablets (Tavanic® 250) were purchased from Hoechst Marion Roussel (Frankfurt, Germany).

2.2. Apparatus

The CE system consisted of a Beckman P/ACE 2100 instrument with a laser-induced fluorescence detector including a 520 nm interference filter (Beckman Instruments, Munich, Germany), to select the most appropriate emission wavelength.

Fluorescence excitation was provided by an Omnichrome HeCd-laser (Series 74, Model No. 3074-EOS-A01, Laser 2000, Wessling, Germany) with 20 mW and a wavelength of 325 nm. The laser was connected to the LIF detector of the CE system by an optical fibre (Omnichrome POS FDS-A$\frac{1}{2}$ Laser 2000, Wessling, Germany). Data collection and processing were performed using System Gold™ software, version 7.11 (Beckman).

An untreated fused-silica capillary of 50 μm I.D.×375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) with a total length of 37 cm and a detection length of 30 cm was used for the separation.

For pH measurement, a digital pH meter (pH522, Fa. WTW, Weilheim, Germany) with an electrode combination (SenTix 50, Fa. WTW, Weilheim, Germany) was used.

An ultrasonic bath (Sonorex TK52, Bandelin Electronic, Berlin, Germany) was used for the preparation of the stock solutions.

2.3. Preparation of reagents, stock- and reference solutions

Phosphoric acid (0.1 *M*) was made by dissolving 1.1529 g orthophosphoric acid (85%) in bidistilled water to a final volume of 100 ml. Phosphoric acid (0.02 *M*) was prepared by diluting one part of 0.1 *M* phosphoric acid with four parts of water. Sodium dihydrogen phosphate solution (0.02 *M*) was made by dissolving 0.2760 g of sodium dihydrogen phosphate monohydrate in bidistilled water to a final volume of 100 ml.

The background electrolyte (BGE) was prepared from 0.1 *M* phosphoric acid adjusted to pH 2.0 by dropwise addition of triethylamine (TEA). Sulfobutyl β-cyclodextrin was dissolved in this plain phosphate–TEA buffer at a concentration of 0.65 mg/ml (0.3 m*M*).

For the preparation of the stock solutions of OFLX, DMOF and OFNO, each compound was dissolved by ultrasonic treatment in 0.02 *M* phosphate buffer, pH 2.0 (made from 0.02 *M* phosphoric acid and 0.02 *M* sodium dihydrogen phosphate solution) to afford concentrations of about 1 mg/ml for OFLX and 0.05 mg/ml for DMOF and OFNO.

The stock solution of quinine sulfate (I.S.) was prepared by dissolving the I.S. in 0.02 *M* phosphoric acid at a concentration of 1 mg/ml.

The stock solutions of OFLX, DMOF and OFNO were diluted with blank urine just before analysis, up to the concentration levels required for calibration and validation of the method. To 5 ml of every dilution, 50 μl of I.S. solution were added afterwards. During the analysis, all reference solutions were stored in brown glass vials to protect the analytes from light.

2.4. Assay performance

Half an hour before starting a series, the laser was ignited and the capillary was rinsed with a freshly prepared running buffer. All rinsing procedures were performed at a pressure of 20 p.s.i. (1 p.s.i.= 6894.76 Pa). Three pre-runs with an aqueous standard solution containing 10 μg/ml of OFLX, DMOF and OFNO in 0.02 *M* phosphate buffer, pH 2.0, were performed to equilibrate the system and to check the actual separation quality. A typical electropherogram of this standard solution is shown in Fig. 2.

Prior to the injection of each sample solution, the capillary was rinsed for 3 min with running buffer. Urine samples and reference solutions were injected hydrodynamically for 5 s at 0.5 p.s.i. (equivalent to an injected sample volume of 7.12 nl and an injection plug length of 3.62 mm).

Separation was carried out by applying a voltage of 25 kV with normal polarity (anode at the injection side and cathode at the detection side of the capillary), resulting in an electric field strength of 676 V/cm and a current of approximately 95 μA. The temperature of the capillary was kept at 20°C during the separation by a liquid cooling system.

After each run, the capillary was rinsed with methanol for 2 min, water for 30 s, 0.1 *M* NaOH for 2 min and again with water for 30 s.

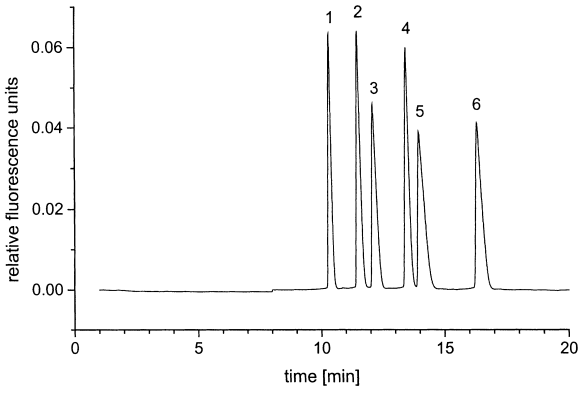

Fig. 2. Electropherogram of an aqueous standard solution containing 5 μg/ml of *S*-(−)-OFLX (2), *R*-(+)-OFLX (5), *S*-DMOF (1), *R*-DMOF (3), *S*-OFNO (4) and *R*-OFNO (6); electrophoretic conditions: 30/37 cm capillary; applied voltage, 25 kV; BGE: 100 m*M* phosphate–TEA buffer, pH 2.0, containing 0.65 mg/ml sulfobutyl β-cyclodextrin.

The running buffer was exchanged after 12–15 runs. At the end of a series, the capillary was rinsed with methanol for 15 min, 0.1 *M* NaOH for 30 min, water for 5 min and was then dried with nitrogen for 5 min.

2.5. Calibration

Samples for the calibration were obtained as described in Section 2.3 at eight concentration levels in the range of 0.25 to 35 μg/ml (0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 35.0 μg/ml) for each OFLX enantiomer and 0.1 to 2.5 μg/ml (0.1, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0 and 2.5 μg/ml) for each metabolite enantiomer. Every calibration sample was injected and analyzed three times, and the mean corrected peak areas were evaluated. Calibration curves according to the I.S. method were obtained by plotting the concentration on the *x*-axis against corrected peak area ratios on the *y*-axis and calculating the optimal calibration functions by linear regression.

2.6. Validation

For validation of the method, parameters such as accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), linearity, working range and selectivity were investigated.

To evaluate accuracy and precision, four spiked urine samples at four concentration levels over the whole calibrated concentration range were prepared as described in Section 2.3 using separately weighed stock solutions. The concentration levels were 35, 10, 2.5 and 0.25 μg/ml for each OFLX enantiomer and 2.5, 1.5, 0.5 and 0.1 μg/ml for the DMOF and OFNO enantiomers. Each of these samples was analyzed six times within one day, to determine the intra-day accuracy and precision; the whole procedure was performed three times on three different days to evaluate inter-day variability.

Precision and accuracy were calculated as the relative standard deviation and the percentage deviation of the observed concentration from the theoretical concentration, respectively. Both should be less than 10% over the complete calibration range and less than 20% near the LOQ [22]. The LOQ was also determined by these experiments.

The selectivity, i.e., the ability of the method to

determine the enantiomers of OFLX and its metabolites in the presence of endogenous compounds was confirmed through the analysis of a blank urine sample.

2.7. Collection and analysis of urine samples

Urine samples from a healthy, 63-year-old, male volunteer were collected directly before (blank urine) and at certain time intervals (0–1, 1–3, 3–6, 6–9 and 9–20 h) after the oral administration of 200 mg of *R,S*-(±)-ofloxacin as a tablet. The volume of urine was measured directly after collection. After a washing out time of more than 3 weeks, 100 mg of *S*-(−)-ofloxacin were orally administered to the same volunteer as an equivalent of a pulverized tablet and again urine samples were collected.

The urine samples were stored at −18°C until analysis. On the day of analysis, the samples were allowed to thaw at room temperature and were filtered through a folded filter to remove insoluble particles. To 5 ml of every urine sample were added 50 μl of I.S. solution before injection into the capillary. Like the reference solutions, all urine samples were stored in brown glass vials during the analysis.

Every sample was analyzed twice and the mean value of the relative corrected peak areas was taken to calculate the concentrations of the analytes.

3. Results and discussion

3.1. Electrophoretic conditions

Electrophoretic conditions were optimized, in particular with regard to buffer composition and pH value, concentration of the cyclodextrin used, applied voltage and selected emission wavelength.

OFLX and its metabolites have zwitterionic properties by virtue of their carboxyl group being deprotonated at basic pH values and their amino function in position 4 of the piperazine ring being positively charged under acidic conditions. Thus, an electrophoretic separation of the analytes should be possible at nearly all pH values except at their isoelectric points, which is 7.4 for OFLX. As the ability of these compounds to fluoresce at basic pH values is low, pH 2.0 was chosen for the separation.

The addition of triethylamine to the background electrolyte results in an improvement in peak symmetry and resolution [1]. Furthermore, due to their relatively low electrophoretic mobility, triethylammonium ions enable the employment of high voltage, thus resulting in short analysis times without the negative effect of high currents [23].

Different cyclodextrins, such as native α-, β- and γ-cyclodextrin and several of their methylated, hydroxypropylated and sulfoalkylated derivatives, have been investigated with regard to the simultaneous separation and enantioseparation of all three enantiomeric pairs. Sulfobutyl β-cyclodextrin was found to be the most efficient chiral selector for this purpose. Due to the presence of sulfonic acid groups, this cyclodextrin derivative is negatively charged even at pH 2. Due to its charge, the sulfobutyl β-cyclodextrin moves in the opposite direction to the analytes during electrophoresis. This results in very efficient separations even at low concentrations of the chiral selector [24,25]. The cyclodextrin concentration of 0.65 mg/ml is of crucial importance for the separation, because even slight variations in this concentration result in overlapping peaks.

The applied voltage chosen was as high as possible in order to obtain short migration times, but was limited by the heating of the capillary, which could not be compensated for by the liquid cooling system at a current of more than 100 μA.

The fluorescence spectrum of OFLX in phosphate buffer at pH 2 (λ_{ex}=325 nm) shows a maximum at 505 nm. Since an emission light filter with a transmission maximum at 505 nm was not commercially available, a 520-nm interference filter with a half bandwidth of 10 nm was used for LIF detection.

3.2. Electropherograms

Under the conditions described, the migration times of the analytes were 11.5 and 14.0 min for *S*-(−)- and *R*-(+)-OFLX (migration time ratio (*R*/*S*)=1.22), 10.5 and 12.3 min for *S*- and *R*-DMOF (migration time ratio (*R*/*S*)=1.17) and 13.5 and 16.3 min for *S*- and *R*-OFNO (migration time ratio (*R*/*S*)=1.21). The migration time of the internal standard (quinine) was 3.8 min.

Representative electropherograms of blank urine and spiked urine samples containing all analytes of interest are shown in Fig. 3. Comparison of the spiked urine samples with the blank urine shows the high selectivity of the method, as no matrix peaks interfere with the determination. A baseline separation of all peaks indicates excellent efficiencies of the separations with high resolution factors.

The peaks could be identified by spiking with (racemic) standard substances. The migration order of the enantiomers was evaluated in a further experiment by spiking with a urine sample received from a volunteer dosed with pure *S*-(−)-ofloxacin. As it has been already shown that no racemisation or chiral inversion takes place in humans [13], this urine sample (Fig. 4B) would be expected to contain only the *S*-isomers of the three analytes.

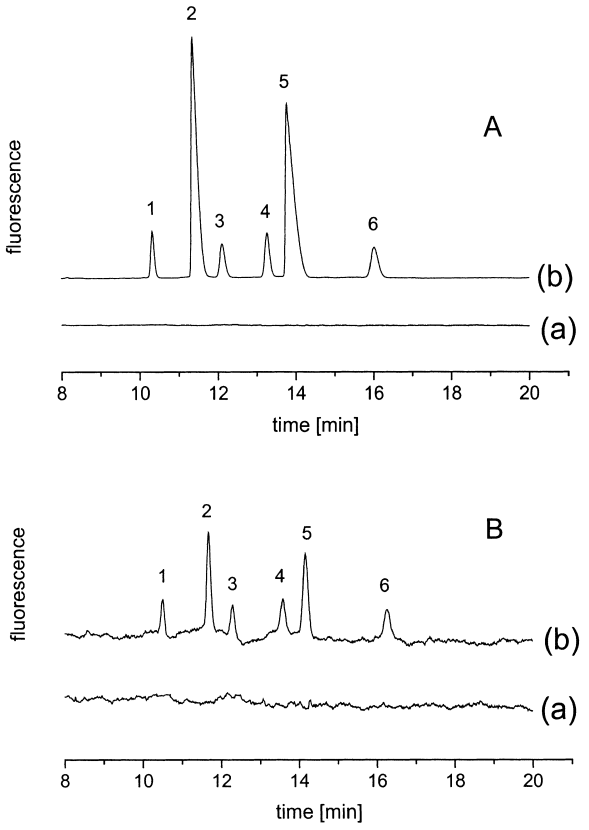

Fig. 3. Electropherograms of (A) blank urine (a) and a spiked urine sample (b) containing 10 μg/ml of *S*-(−)-OFLX and *R*-(+)-OFLX and 1.5 μg/ml of each metabolite enantiomer and (B) blank urine (a) and a spiked urine sample at the LOQ (b) with 250 ng/ml of each OFLX enantiomer and 100 ng/ml of each metabolites' enantiomers (1=*S*-DMOF, 2=*S*-(−)-OFLX, 3=*R*-DMOF, 4=*S*-OFNO, 5=*R*-(+)-OFLX, 6=*R*-OFNO). For electrophoretic conditions, see Fig. 2.

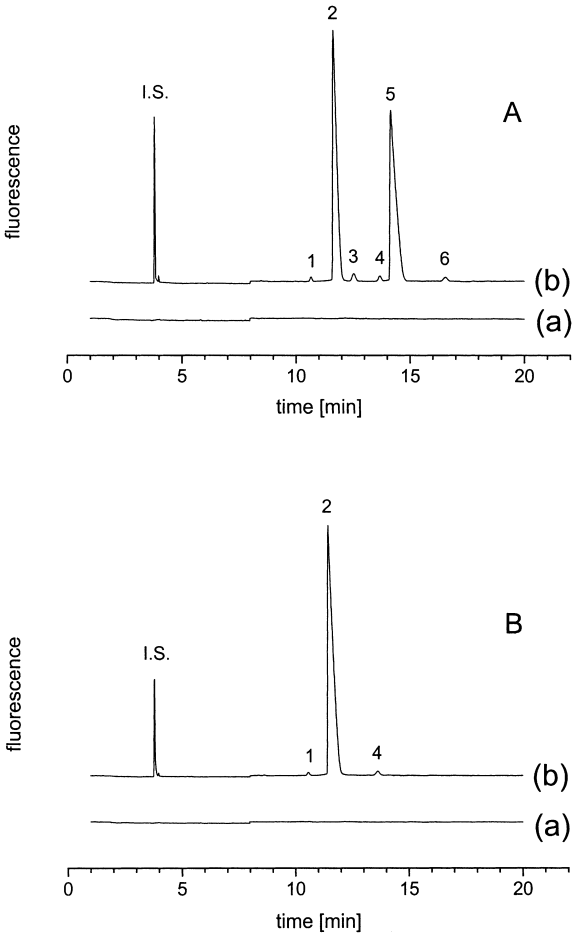

Fig. 4. Electropherograms of (A) blank urine (a) and human urine 20 h after oral administration of 200 mg of (±)-ofloxacin (b) and (B) blank urine (a) and human urine 8 h after oral administration of 100 mg of levofloxacin (b) (I.S.=quinine; 1=*S*-DMOF, 2=*S*-(−)-OFLX, 3=*R*-DMOF, 4=*S*-OFNO, 5=*R*-(+)-OFLX, 6=*R*-OFNO). For electrophoretic conditions, see Fig. 2.

3.3. Assay validation

The method was validated in the concentration range of 0.25 to 35 μg/ml for *S*-(−)-OFLX and

R-(+)-OFLX, and of 0.1 to 2.5 μg/ml for *S*- and *R*-DMOF and *S*- and *R*-OFNO. The LOQ was 250 ng/ml for the OFLX enantiomers and 100 ng/ml for the metabolites' enantiomers (Fig. 3B); the LOD was 25 ng/ml for all compounds (signal-to-noise ratio 3:1).

Calibration functions for all six analytes with full information about slope, intercept, number of data points and correlation coefficients are given in Table 1. In order to obtain satisfactory accuracy values even at low concentrations of the analytes, calibration functions were divided into two parts, the first corresponding to the lower and the second to the higher concentrations. Within each part, linearity was good, with correlation coefficients consistently greater than 0.997.

The fluorescence intensities (and, consequently, the slope values of the calibration curves) were always higher for the *S*-enantiomers than for the *R*-enantiomers. It appears that the diastereomeric cyclodextrin complexes of the *S*-configured isomers of all three compounds exhibit a higher degree of fluorescence than those of the corresponding *R*-isomers. This observation can be confirmed by the electropherogram shown in Fig. 2. A similar phenomenon had been observed previously for the enantiomers of zopiclone [26]. In contrast to zopiclone, however, the peak height and corrected peak area of the slower-migrating enantiomers of OFLX and its metabolites are lower than those of the faster-migrating enantiomers.

The results of intra- and inter-day reproducibility experiments are presented in Tables 2–4. Precision (relative standard deviation) and accuracy values (percentage deviation of the found concentration from the nominal concentration) were consistently less than 10% for the highest three concentration levels and within 16% for the concentration level at the LOQ. Thus, the method fulfils the requirements for bioassays.

3.4. Application to urine samples

The method was applied to urine samples from a human volunteer. A typical electropherogram of a urine sample collected from the volunteer 20 h after oral administration of 200 mg of *R,S*-(±)-ofloxacin is shown in Fig. 4A.

Table 5 shows the urinary concentrations of the enantiomers of OFLX and its metabolites and the concentration ratios (*S*/*R*) 1, 3, 6, 9 and 20 h after the intake of the racemic drug. As expected, urinary concentrations of *S*-(−)-OFLX in the earlier period after dosing were lower than those of the *R*-(+)-form, which changed at later time periods. This is related to the lower elimination rate and renal clearance of *S*-(−)-OFLX in comparison to *R*-(+)-OFLX [12,27]. The cumulative urinary excretion of *S*-(−)-OFLX and *R*-(+)-OFLX shown in Fig. 5A illustrates this slight differences between the two enantiomers.

Regarding the cumulative urinary excretion pro-

Table 1
Calibration functions and correlation coefficients

Compound	Range (μg/ml)	Calibration function[a]	Standard error (slope)	Standard error (intercept)	Correlation coefficient (r)	Number of data points (n)
S-(−)-ofloxacin	0.25–5.00	$y=0.1559\ x-0.0072$	0.0013	0.0032	0.9997	5
	5.00–35.0	$y=0.1606\ x-0.0657$	0.0037	0.0832	0.9997	4
R-(+)-ofloxacin	0.25–5.00	$y=0.1261\ x-0.0053$	0.0009	0.0024	0.9998	5
	5.00–35.0	$y=0.1375\ x-0.0841$	0.0033	0.0734	0.9996	4
S-desmethyl ofloxacin	0.10–1.00	$y=0.1198\ x-0.0010$	0.0015	0.0008	0.9993	5
	1.00–2.50	$y=0.1474\ x-0.0286$	0.0051	0.0095	0.9993	4
R-desmethyl ofloxacin	0.10–1.00	$y=0.1040\ x-0.00004$	0.0013	0.0007	0.9997	5
	1.00–2.50	$y=0.1193\ x-0.0138$	0.0044	0.0080	0.9993	4
S-ofloxacin *N*-oxide	0.10–1.00	$y=0.1216\ x-0.0015$	0.0024	0.0013	0.9983	5
	1.00–2.50	$y=0.1385\ x-0.0176$	0.0061	0.0113	0.9976	4
R-ofloxacin *N*-oxide	0.10–1.00	$y=0.1078\ x-0.0017$	0.0017	0.0009	0.9989	5
	1.00–2.50	$y=0.1116\ x-0.0067$	0.0048	0.0087	0.9997	4

[a] x=concentration of the compound; y=corrected peak area (compound)/corrected peak area (I.S.).

Table 2
Intra- and inter-day accuracy and precision data for the determination of *S*-(−)- and *R*-(+)-ofloxacin

Nominal concentrations (μg/ml) of *S*-(−)-ofloxacin/*R*-(+)-ofloxacin				
	0.25/0.25	2.5/2.5	10/10	35/35
Concentration found (arithmetic mean value) (μg/ml)				
Day 1 (n=6)	0.245/0.248	2.29/2.32	10.85/10.27	32.84/33.29
Day 2 (n=6)	0.240/0.236	2.37/2.33	9.64/9.75	34.81/34.96
Day 3 (n=6)	0.264/0.269	2.26/2.32	9.34/9.43	32.85/31.92
Inter-day (n=18)	0.250/0.251	2.31/2.33	9.94/9.82	33.50/33.39
Accuracy (arithmetic mean value) (%)				
Day 1 (n=6)	97.9/99.2	91.5/93.0	108.5/102.7	93.8/95.1
Day 2 (n=6)	95.9/94.4	95.0/93.3	96.4/97.5	99.5/99.9
Day 3 (n=6)	105.8/107.6	90.3/93.0	93.4/94.3	93.8/91.2
Inter-day (n=18)	99.9/100.4	92.3/93.1	99.4/98.2	95.7/95.4
Precision (arithmetic mean value) (%)				
Day 1 (n=6)	4.20/8.19	2.69/2.80	3.82/3.71	4.38/5.14
Day 2 (n=6)	5.54/4.36	2.75/1.27	1.32/0.84	0.80/0.83
Day 3 (n=6)	7.03/4.29	1.50/0.94	5.09/5.17	1.12/1.13
Inter-day (n=18)	7.67/8.69	3.18/1.75	7.88/5.18	3.78/4.85

files of the metabolites' enantiomers (Fig. 5B–C), in particular *S*- and *R*-DMOF, these differences are much more distinctive. Thus, the ratios of the enantiomers (*S*/*R*) are always lower than unity; in the case of DMOF, they are even lower than 0.5 in the first 20 h.

It can be concluded that either the formation of the metabolites is stereoselective in the way that *S*-(−)-OFLX is metabolized less than *R*-(+)-OFLX, or that the renal clearance values of the *S*-enantiomers of the two metabolites are much lower than those of the *R*-isomers with the consequence of later excretion.

4. Conclusion

A sensitive and selective CE–LIF method for the determination of the enantiomers of ofloxacin and its metabolites, desmethyl ofloxacin and ofloxacin *N*-oxide in human urine has been established. Laser-

Table 3
Intra- and inter-day accuracy and precision data for the determination of *S*- and *R*-desmethyl ofloxacin

Nominal concentrations (μg/ml) of *S*-desmethyl ofloxacin/*R*-desmethyl ofloxacin				
	0.1/0.1	0.5/0.5	1.5/1.5	2.5/2.5
Concentration found (arithmetic mean value) (μg/ml)				
Day 1 (n=6)	0.100/0.092	0.504/0.496	1.61/1.57	2.31/2.30
Day 2 (n=6)	0.096/0.090	0.524/0.515	1.57/1.59	2.48/2.45
Day 3 (n=6)	0.102/0.103	0.525/0.532	1.52/1.53	2.46/2.42
Inter-day (n=18)	0.099/0.095	0.518/0.514	1.57/1.56	2.42/2.39
Accuracy (arithmetic mean value) (%)				
Day 1 (n=6)	99.6/92.1	100.9/99.2	107.3/104.8	92.4/91.9
Day 2 (n=6)	96.0/90.1	104.9/102.9	104.3/105.9	99.1/97.8
Day 3 (n=6)	102.1/103.3	105.1/106.4	101.7/101.7	98.5/96.9
Inter-day (n=18)	99.2/95.1	103.6/102.8	104.4/104.1	96.7/95.5
Precision (arithmetic mean value) (%)				
Day 1 (n=6)	4.75/8.96	3.25/4.28	2.92/4.61	4.47/3.52
Day 2 (n=6)	9.13/7.00	4.93/0.99	1.13/1.26	2.28/0.87
Day 3 (n=6)	8.29/13.43	1.81/2.15	6.82/5.35	1.28/1.49
Inter-day (n=18)	7.67/11.69	3.89/3.92	4.77/4.30	4.43/3.59

Table 4
Intra- and inter-day accuracy and precision data for the determination of *S*- and *R*-ofloxacin *N*-oxide

Nominal concentrations (μg/ml) of *S*-ofloxacin *N*-oxide/*R*-ofloxacin *N*-oxide				
	0.1/0.1	0.5/0.5	1.5/1.5	2.5/2.5
Concentration found (arithmetic mean value) (μg/ml)				
Day 1 (n=6)	0.115/0.103	0.467/0.455	1.62/1.52	2.39/2.26
Day 2 (n=6)	0.109/0.103	0.506/0.511	1.54/1.57	2.61/2.46
Day 3 (n=6)	0.113/0.111	0.526/0.498	1.53/1.55	2.40/2.39
Inter-day (n=18)	0.112/0.105	0.500/0.488	1.56/1.54	2.47/2.37
Accuracy (arithmetic mean value) (%)				
Day 1 (n=6)	114.6/102.7	93.4/91.0	107.8/101.0	95.8/90.4
Day 2 (n=6)	109.3/103.0	101.2/102.2	102.5/104.7	104.3/98.5
Day 3 (n=6)	113.3/110.6	105.2/99.6	101.9/103.3	96.2/95.7
Inter-day (n=18)	112.4/105.4	99.9/97.6	104.1/103.0	98.8/94.9
Precision (arithmetic mean value) (%)				
Day 1 (n=6)	5.94/9.25	3.70/3.72	8.16/5.67	6.96/5.91
Day 2 (n=6)	9.66/14.12	3.92/3.98	1.71/2.13	1.69/2.74
Day 3 (n=6)	15.88/7.01	1.85/2.11	5.21/6.62	1.50/0.75
Inter-day (n=18)	10.83/10.62	6.00/6.07	6.14/5.09	5.81/5.10

induced fluorescence detection provided sufficient sensitivity to determine low concentrations of the metabolites' enantiomers by direct injection of urine into the capillary. This makes the performance of the assay very simple, because no sample preconcentration or extraction procedure is required. Moreover, acceptable levels of accuracy and precision of the assay have been shown.

This method has been utilized in the analysis of urine samples collected from a human volunteer who was administered (±)-ofloxacin. Stereoselective differences in the pharmacokinetic properties of the unchanged drug and its metabolites have been observed.

CE–LIF, a relatively new analytical separation technique, combines the advantages of the simple performance of chiral separations by CE with the excellent sensitivity and selectivity obtained by using LIF detection.

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Table 5
Concentrations of the enantiomers of OFLX and its metabolites and concentration ratios (S/R) in human urine after oral administration of 200 mg of (±)-OFLX

Time interval	0–1 h	1–3 h	3–6 h	6–9 h	9–20 h
[*S*-OFLX] (μg/ml)	12.41	25.00	18.34	16.68	14.02
[*R*-OFLX] (μg/ml)	13.09	27.21	19.65	17.27	13.46
[*S*-OFLX]/[*R*-OFLX]	0.95	0.92	0.93	0.97	1.04
[*S*-DMOF] (μg/ml)	0	0.179	0.164	0.167	0.207
[*R*-DMOF] (μg/ml)	0	0.432	0.390	0.426	0.488
[*S*-DMOF]/[*R*-DMOF]	–	0.41	0.42	0.39	0.42
[*S*-OFNO] (μg/ml)	0	0.252	0.278	0.274	0.231
[*R*-OFNO] (μg/ml)	0	0.287	0.371	0.335	0.245
[*S*-OFNO]/[*R*-OFNO]	–	0.88	0.75	0.82	0.94

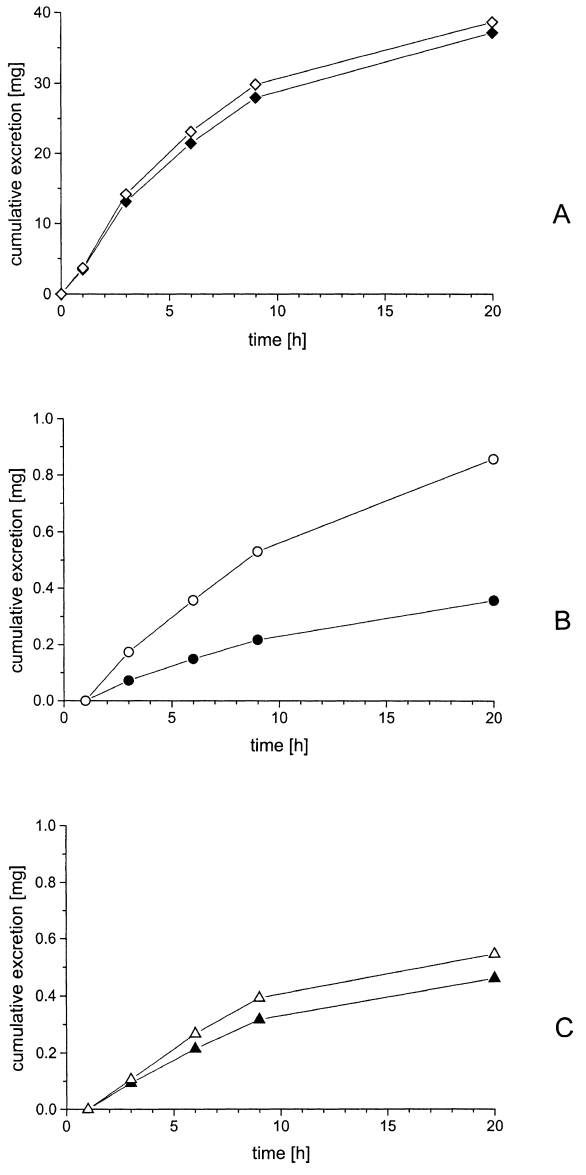

Fig. 5. Cumulative excretion of the enantiomers of OFLX (A), DMOF, calculated as OFLX (B), and OFNO, calculated as OFLX (C), after oral administration of 200 mg of (±)-ofloxacin [♦=*S*-(−)-OFLX, ◇=*R*-(+)-OFLX, ●=*S*-DMOF, ○=*R*-DMOF, ▲=*S*-OFNO, △=*R*-OFNO].

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