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Capillary electrophoresis with laser-induced fluorescence: a routine method to determine moxifloxacin in human body fluids in very small sample volumes

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Abstract

The feasibility of capillary electrophoresis with HeCd laser-induced fluorescence detection as a validated routine method for bioanalytical analysis is reported. Method evaluation, validation and results of the determination of moxifloxacin (BAY 12-8039), a new antimicrobially active 8-methoxy-quinolone, in plasma and microdialysate are described. After a one step sample preparation the samples can be injected directly into the capillary. The volume of microdialysate and plasma, respectively, needed for more than 50 injections is only 10 μl and 20 μl. Total run time is less than 7 min using a 27 cm capillary on commercial instrumentation. An analysis time of less than 1 min was shown to be possible, however it could not be used routinely since appropriate instrumentation was not available. Evaluation is based on the relative corrected peak area (analyte/I.S.). The method's dynamic range comprises three orders of magnitude (plasma: 2.5–5000 μg/l; microdialysate: 5–5000 μg/l). Validation according to international guidelines yielded data on accuracy and precision of the method throughout the entire working range of inter-day precision: plasma <6%, microdialysate <5% and inter-day accuracy: plasma <2%, microdialysate <4%. The crossvalidation with an existing HPLC method utilizing clinical study samples shows linear correlation. In view of its adequate sensitivity and high selectivity capillary electrophoresis with laser-induced fluorescence is a very versatile tool in pharmacokinetic studies of quinolones, especially in situations with limited sample volumes: e. g. pediatrics, patients at risk, animal-, microdialysis- and tissue-kinetic studies. Validation parameters and other features, like high sample throughput and robustness, are comparable to or even better than HPLC. Further necessary improvements of the capillary electrophoresis with laser-induced fluorescence instrumentation (autosampler, vials, parallel capillaries) and its use in bioanalytical routine analysis are discussed.

Keywords: Moxifloxacin; Microdialysate

1. Introduction

Capillary electrophoresis (CE) is a technique that is rapidly gaining popularity in pharmaceutical quality control [1–3]. The advantages of short analysis times, small injection volumes (a few nanoliters) [4] and low amounts of solvents make this method also attractive for bioanalytical assays in pharmaceutical industry and hospitals. Apart from validation according to international guidelines [5–7] there are four

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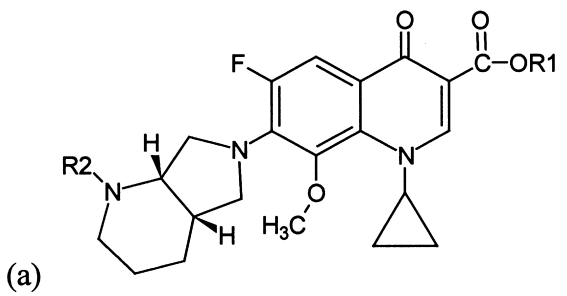
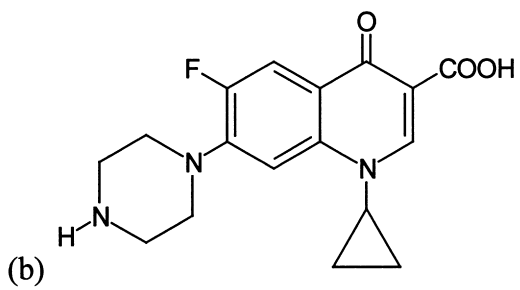
important points to consider for application in clinical studies: adequate lower limit of quantification (LOQ), large dynamic range, high sample throughput (practicality) and small sample volume requirements for analysis.

In the past, the use of CE in clinical studies was restricted by high detection limits [8,9]. These could be lowered by implementing laser-induced fluorescence (LIF)-detection. Using a 20 mW HeCd-laser with a wavelength of 325 nm, CE–LIF can be applied to all compounds that can be excited to native fluorescence around this wavelength [10–13]. However, it remains to be investigated if the HeCd-laser with its fixed wavelength is also applicable to analytes that do not have their excitation maximum at or close to 325 nm.



(a)

	R1	R2
Moxifloxacin	- H	- H
M1	- H	- SO_3H
M2	- Gluc	- H



(b)

Fig. 1. Structures of (a) moxifloxacin and its metabolites and (b) ciprofloxacin (I.S.).

Moxifloxacin (Fig. 1), a new enantiomerically pure 8-methoxy-quinolone, is such a compound, possessing a fluorophore with an excitation maximum at 296 nm (Fig. 2). It has potent antimicrobial activity including both gram-negative and gram-positive bacteria and anaerobes [14]. Preclinical studies showed excellent antimicrobial efficacy [15]. With clinical studies in man ongoing [16], an analytical technique was required to measure more than 1000 samples with volumes <50 μl. The main objective of the present work was therefore to develop a CE–LIF assay for this drug, and assess its utility for routine use.

In the frame work of this assessment the parameters (e.g. dynamic ranges) of CE–LIF and HPLC–fluorescence methods were compared to judge upon an easy use of the assays in clinical studies.

To fully explore the potential of CE–LIF it has been investigated, whether a simple and rapid sample preparation is sufficient. High sample throughput can only be achieved if all stages during analytical processes from sample pretreatment to analysis and data evaluation are streamlined. The attempt will be reported to minimize the time required for sample work-up to open CE to parallel analysis [17,18] and short retention times to less than 1 min.

2. Experimental

2.1. Chemicals and material

All reagents used were of analytical grade unless otherwise indicated.

Moxifloxacin and ciprofloxacin were used as certified reference compounds (Bayer, Wuppertal, Germany) for quantitative analysis. Since both standards were hydrochlorides but found as betaine in biological fluids, all concentrations were calculated based on the betaine (M_r moxifloxacin=401.4/M_r ciprofloxacin=331.5).

Acetonitrile (HPLC grade) and orthophosphoric acid were purchased from Riedel de Haen (Seelze, Germany), sodium hydroxide and sodium dihydrogen phosphate from Merck (Darmstadt, Germany). Triethylamine was obtained from Fluka

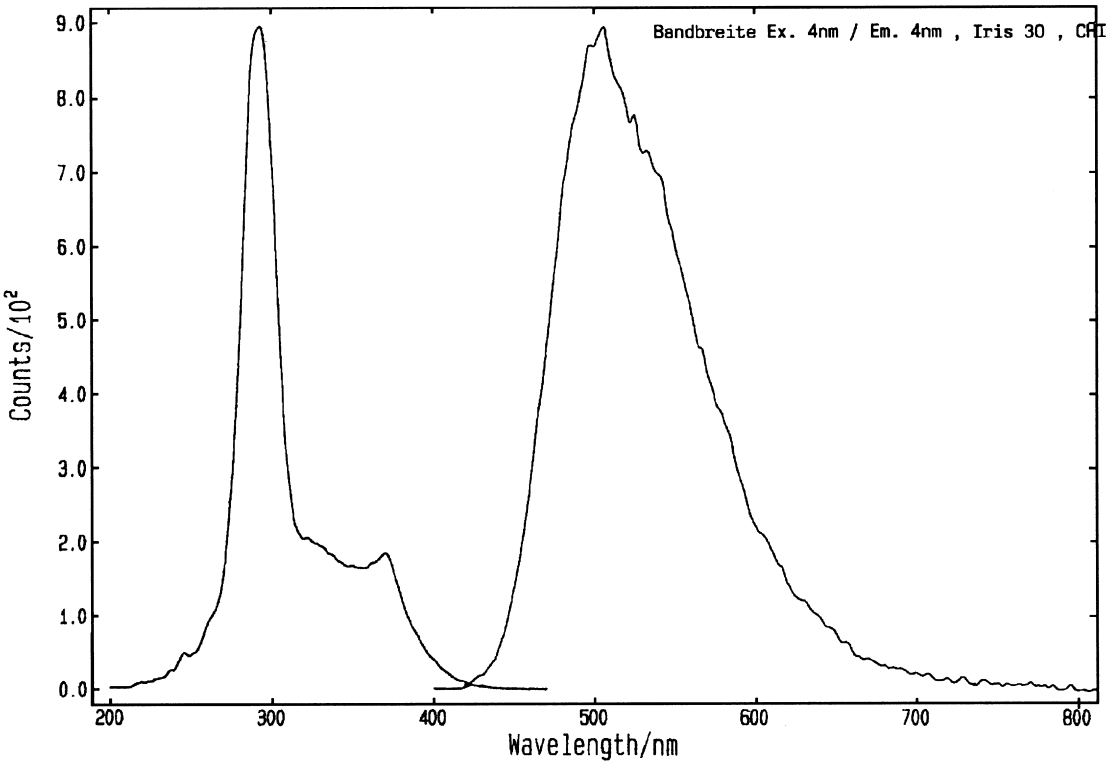


Fig. 2. Absorption and emission spectrum (λ_{ex}=296 nm) of moxifloxacin (2 mg/l in phosphate buffer pH=2.0).

(Buchs, Switzerland). Water was purified using the Milli Q system (Millipore Waters, Eschborn, Germany). Nitrogen was purchased from Messer Griessheim (Griessheim, Germany).

Blank plasma was either obtained from fasted healthy volunteers or purchased from the German Red Cross (DRK, Hagen, Germany).

The vacuum filtration unit and 0.45 μm filters were purchased from Millipore.

2.2. Preparation of reagents and stock solutions

Moxifloxacin was dissolved in 0.1 *M* phosphate buffer pH=4.0 to a concentration of 100 mg/l. Various working solutions with concentrations in the range between 100 mg/l and 0.05 mg/l were prepared.

Ciprofloxacin (internal standard, I.S.) was dissolved in 0.1 *M* phosphoric acid to a concentration of 100 mg/l.

For plasma analysis the precipitation reagent with I.S. was prepared by diluting the ciprofloxacin stock solution 1:99 (v/v) with a mixture (v/v) of nine parts acetonitrile and one part 0.1 *M* phosphoric acid.

For microdialysate analysis the dilution reagent with I.S. was obtained by diluting the ciprofloxacin stock solution 1:99 (v/v) with acetonitrile.

Electrophoresis buffer was prepared from phosphoric acid adjusted to the required pH with (concentrated) triethylamine. 60 m*M* phosphoric acid at a pH of 2.2 was used for plasma samples and 80 m*M* phosphoric acid at a pH of 2.0 for microdialysate samples. The solutions were passed through a 0.45 μm filter.

2.3. Sample pretreatment plasma

Fifty microlitres (or less) of plasma were diluted 1:3 (v/v) with the precipitation reagent containing I.S. The solution was vortex-mixed for 5 s and

centrifuged for 10 min at 3200 *g* to separate the precipitated proteins. The supernatant was injected into the capillary.

2.4. Sample pretreatment microdialysate

10 μl of microdialysate were diluted with 20 μl of the diluting reagent containing I.S. The mixture was injected hydrodynamically into the capillary.

2.5. Instrumentation

CE analysis was performed on a Beckman P/ACE 5010 instrument with a laser-induced fluorescence detector (Beckman Instruments, Munich, Germany). The instrument was equipped with a cooled sample holder. It was mandatory to use cryogenic vials to prevent evaporation of acetonitrile. The autosampler temperature was kept at 10°C using a Haake D8 water bath (Haake, Karlsruhe, Germany). Lower temperatures occasionally yielded in current breakdowns. The CE–LIF instrument was run in an air-conditioned room (20–25°C). Fluorescence excitation was provided by an Omnichrome HeCd-laser (3074-20M, Laser 2000, Wessling, Germany) with 20 mW and 325 nm excitation wavelength. Laser and detector were connected by an optical fiber (Omnichrome POS FDS - A 1/2, Laser 2000, Wessling, Germany). The energy measurable at the end of the detector's fiber optics was 7 mW. Emission light was filtered through a 520 nm filter with a half bandwidth of 10 nm. Data were collected and integrated using the software Gold 8.10 (Beckman, Munich, Germany).

A fused-silica capillary of dimension 27 cm×50 μm with an extended light path (=bubble cell) (Hewlett–Packard, Waldbronn, Germany) was employed [19]. Capillaries were initially preconditioned, prior to their first use, by rinsing for 60 min with 0.1 *M* NaOH to regenerate surface silanol groups. As bubble cells differ and variability may occur as a result of fitting the capillaries, the LOQ (and LOD) were checked by re-validation. One capillary was used only for one matrix. Prior to storage the capillary was flushed 10 min with 0.1 *M* phosphoric acid, 5 min with water and then with air/nitrogen.

The liquid cooling system of the capillary was set to 20°C.

2.6. Assay

All rinsing stages were carried out with 20 p.s.i. (1 p.s.i.=6894.76 Pa) unless otherwise indicated. Before starting a series the laser was ignited at least 20 min in advance. Three pre-analyses were performed to allow the system to settle.

Prior to each plasma sample the capillary was rinsed 0.8 min with 0.1 *M* NaOH for cleaning purposes. This step could be reduced to 0.3 min analyzing microdialysate. Afterwards the capillary was re-conditioned by rinsing 0.3 min with electrophoresis buffer taken from a buffer vial other than that used during separation.

Injection was performed hydrodynamically for 8 to 12 s at 0.5 p.s.i. During injection the opposite end of the capillary was already located in the electrophoresis buffer vial. As the injection volume was only approx. 20 to 30 nl, repeated injections were possible.

Separation was carried out by applying a voltage of 20 kV (microdialysate: 18 kV) with normal polarity; this corresponds to 714 (666) V/cm. The current is then approx. 85 μA. The migration times are 3.6 (3.9) min for the I.S. and 4.1 (4.6) min for moxifloxacin. With a separation time of 4.5 (5.0) min and taking into account the rinse stages and injection, the total time of analysis per sample is 6.4 (6.3) min.

Evaluation was based on relative corrected peak areas.

2.7. Calibration

Calibration samples were obtained by spiking 19 parts of blank plasma/microdialysate with one part of working solutions (Section 2.2) of moxifloxacin to yield ten concentrations in the range of 2.5 to 5000 μg/l. Quality control samples were prepared in the same way at six levels covering the same range of concentrations. These QC-samples were stored together with the unknown samples in a freezer at −20°C. All samples were processed further as described in Sections 2.3 and 2.4 and analyzed in one sequence.

Calibration curves according to the internal standard method were obtained by plotting concentration versus corrected peak area ratios. Linear regression with a y^{-2} weighting factor resulted in the calibration function for the plasma method with lowest residuals and best curve fit (Concalc software, Institute of Clinical Pharmacology, Bayer, Wuppertal). Log–log iteration was performed for the microdialysate method since a slightly overproportional increase of the signal was observed at higher concentrations.

2.8. Validation

Validation was performed according to the international guidelines for development of bioanalytical assays in human biomatrices [5–7]. Short and long time stability as well as investigations on light and freeze–thaw stability were already performed [20].

To evaluate accuracy and precision six spiked samples at six to seven concentration levels (0, (2.5), 5, 10, 20, 500, 4000 μg/l) of moxifloxacin were analyzed within one day to assess intra-day variability. The procedure was repeated on three days to investigate inter-day precision and accuracy. The LOQ was also determined by these experiments since accuracy should be less than ±10% and precision less than 10% in the working range. Precision near the LOQ was allowed to be ≤20%. The LOD was evaluated in a different experiment.

A total absence of matrix interference was confirmed by analysis of blank plasma and microdialysate (Figs. 3 to 5). In addition the metabolites (Fig. 1) were injected. They did not migrate under the conditions of this method, since an EOF was absent.

CE–LIF results for plasma derived from a clinical study (Section 2.10) were cross-validated with a high-performance liquid chromatography (HPLC) method.

2.9. HPLC method

The cross-validation was performed with an established HPLC-method [20]. A reversed-phase HPLC

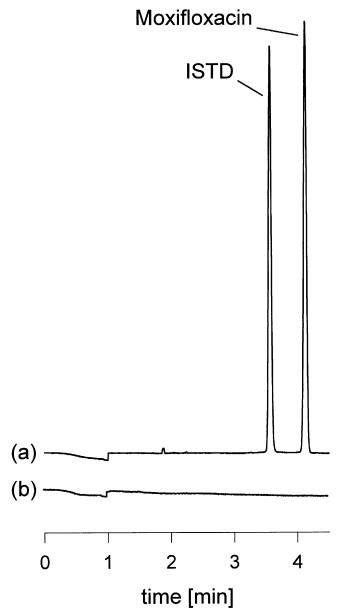


Fig. 3. Electropherograms of (a) human plasma with 1150 μg/l moxifloxacin and I.S. and (b) blank human plasma.

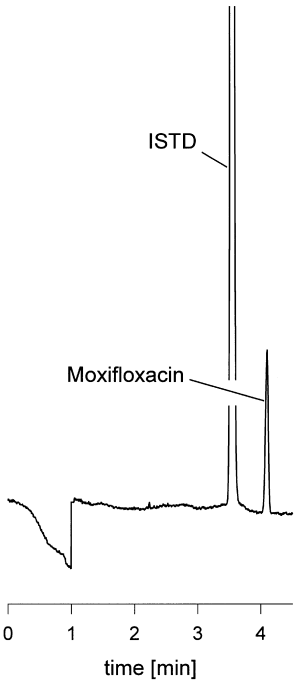


Fig. 4. Electropherogram of human plasma with 50.3 μg/l moxifloxacin and I.S..

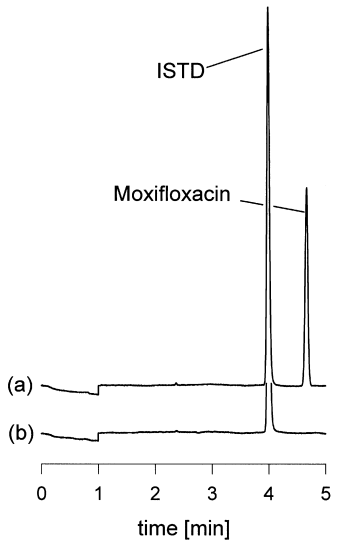


Fig. 5. Electropherograms of (a) microdialysate with 469 μg/l moxifloxacin and I.S. and (b) blank microdialysate with I.S.

was carried out on a HP 1090 instrument (Hewlett–Packard, Waldbronn, Germany) using a Nucleosil 100 C_{18} column. The mobile phases of the gradient consisted of an aqueous solution of 0.01 mol/l tetrabutyl ammonium sulfate and 0.05 mol/l sodium dihydrogen phosphate (pH=2.0) (A) and acetonitrile (B). The analyte was detected using a fluorescence detector HP 1046 A (Hewlett–Packard) with an excitation wavelength of 296 nm and emission at 504 nm.

2.10. Application

Moxifloxacin was determined in plasma and microdialysate samples taken from clinical pharmacological studies. All studies were conducted according to the German Medicines Act, the Helsinki Declaration and Good Clinical Practice rules. Volunteers received 200 mg and 400 mg, respectively, of Moxifloxacin orally and as infusion (1 h), respectively. Blood samples were obtained up to 96 h after administration. Plasma (Sarstedt, Germany) was prepared by centrifugation within 30 min after sampling. Microdialysate was frozen up directly. All samples were stored together with the QC samples at −20°C until analysis.

3. Results

3.1. Capillary electrophoresis

CE–LIF was established for the determination of moxifloxacin in plasma and microdialysate.

Sample pretreatment for CE–LIF analysis required one step as for HPLC. Plasma was precipitated with acetonitrile/0.1 *M* H_3PO_4 containing the I.S. This mixture yielded in a complete, fine precipitation (no inclusions) which could be easily separated by centrifuging. Microdialysate was diluted with acetonitrile containing the I.S., since high ionic strength resulted in very broad peaks and the addition of I.S. was necessary for good precision. Dilution with acetonitrile showed sharper peaks and a higher fluorescence compared to water or other non-ionic solvents. Thus, a two-fold lower LOQ could be achieved.

Using a 20/27 cm fused-silica capillary moxifloxacin and the I.S. (ciprofloxacin) were readily separated within 4.1 min in plasma samples (Figs. 3 and 4) and 4.6 min in microdialysate samples (Fig. 5).

Absence of any interference in all electropherograms was proven by injecting blank plasma and microdialysate (Figs. 3–5) as well as the metabolites. The increase in sensitivity using LIF-detection

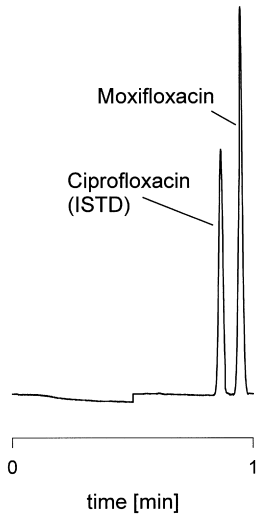


Fig. 6. Electropherogram of plasma spiked with 1000 μg/l ciprofloxacin and 1000 μg/l moxifloxacin manually injected at the short capillary end (7/27 cm).

compared to UV-detection was more than two orders of magnitude under acidic run conditions.

A separation in less than 1 min was achieved for a plasma sample (Fig. 6) by using the 7 cm distance from capillary's end to the detector. The injection was performed manually. All conditions were the same except for a change in polarity. Since automatic, hydrodynamic injection on this side of the capillary is not possible with existing CE–LIF instruments, the short separation time could not be used routinely.

Migration times and separation performance were stable even after more than 2000 injections for each matrix. Data on the long term performance were generated routinely during analysis of unknown samples by analyzing QC samples (at least two replicates at three concentration levels per analytical sequence).

3.2. Validation

The method was validated in a concentration range of 2.5 to 5000 μg/l for plasma and 5 to 5000 μg/l for microdialysate using an internal standard method. The LOQ was 2.5 μg/l for plasma and 5 μg/l for microdialysate; the LOD at 0.5 μg/l based on a signal to noise ratio of 3: 1. Calibration curves were obtained by plotting concentration versus corrected area ratio. Typical calibration results are shown in Fig. 7. The results of a formal validation experiment

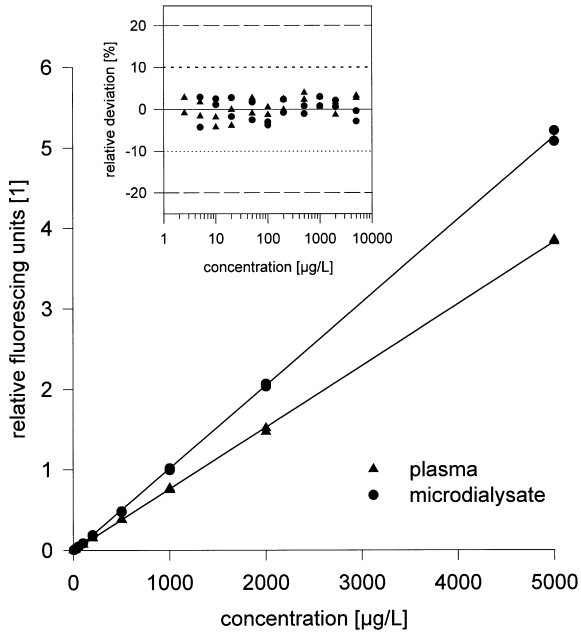


Fig. 7. Typical calibration data for moxifloxacin in plasma and microdialysate: calibration curves and residuals of back calculated concentrations.

Table 1
Intra- and Inter-day accuracy and precision of the determination of moxifloxacin in human plasma

	Nominal concentrations of moxifloxacin in plasma [μg/l] calibration range 2.5–5000					
	2.50	5.00	10.00	20.0	500	4000
Concentration found (arithm. mean value) [μg/l]						
Day 1 (n=6)	2.45	5.37	10.54	21.1	515	4110
Day 2 (n=6)	2.42	4.80	9.45	19.6	477	3930
Day 3 (n=6)	2.33	5.17	10.04	20.1	505	4070
Inter-day (n=18)	2.40	5.11	10.01	20.3	499	4040
Accuracy (arithm. mean value) [%]						
Day 1 (n=6)	98.0	107.4	105.4	105.3	103.0	102.8
Day 2 (n=6)	96.8	95.9	94.5	98.2	95.5	98.3
Day 3 (n=6)	93.4	103.3	99.6	100.3	100.9	101.8
Inter-day (n=18)	96.0	102.2	100.1	101.3	99.8	100.9
Precision (arithm. mean value) [%]						
Day 1 (n=6)	15.5	2.9	4.6	1.9	2.0	1.6
Day 2 (n=6)	8.5	7.6	4.2	3.4	3.0	3.7
Day 3 (n=6)	17.1	12.6	1.9	2.0	2.5	2.1
Inter-day (n=18)	13.5	9.4	5.8	3.8	4.1	3.1

are presented in Table 1 for plasma and Table 2 for microdialysate. Inter-day accuracy and inter-day precision of the method throughout the entire working range (except near LOQ) are 99.8–101.3% with <5.8% precision for plasma and 99.2–103.7% with <4.9% precision for microdialysate.

3.3. Correlation of data from CE–LIF and HPLC

Additionally to the formal validation presented above (Section 3.2) the results of CE–LIF were compared to those determined by HPLC. For this purpose a multiple dose study with eight male, healthy volunteers receiving 200 mg Moxifloxacin orally was analyzed by CE–LIF and HPLC ($n=216$). Fig. 8 shows the excellent correlation ($r^2=0.9897$) between these two methods. The straight line had a slope of 1.00 and an intercept of −8.86. No single data point deviated by more than 14% between HPLC and CE–LIF even at the LOQ.

3.4. Application

The CE–LIF method was used additionally to the HPLC assays in pharmacokinetic studies to analyze moxifloxacin in plasma and microdialysate.

After oral administration of 200 mg moxifloxacin concentrations above the LOQ were measured up to 96 h. Concentrations reached a maximum after 2.5 h (mean t_{max}) and decreased in a biphasic manner with a terminal half-life of approx. 15 h in plasma. Results of eight male, healthy volunteers are presented in the concentration–time profile of Fig. 9. Average and standard deviation of the CE–LIF and HPLC methods were identically. For these eight volunteers a maximum concentration (C_{max}) of 0.95 mg/l in plasma could be determined.

4. Discussion

4.1. Assay conditions

The rationales for choice of buffer were high fluorescence yield at acidic conditions, symmetric peaks and high resolution using triethylamine [21]. Low pHs lead to high currents but short migration times. For the microdialysate assay a higher ionic strength was needed compared to plasma since the ionic strength of the sample was still high even after diluting with acetonitrile. Deionisation was irrelevant due to the small sample volume and a straight forward sample pretreatment.

Table 2
Intra- and Inter-day accuracy and precision of the determination of moxifloxacin in microdialysate

	Nominal concentrations of moxifloxacin in microdialysate [μg/l] calibration range 5–5000					
	5.00	10.00	20.0	50.0	500	4000
Concentration found (arithm. mean value) [μg/l]						
Day 1 ($n=6$)	4.50	9.69	20.0	50.6	513	4240
Day 2 ($n=6$)	5.04	10.38	19.4	48.7	521	4020
Day 3 ($n=6$)	4.46	9.85	20.1	49.7	522	4100
Inter-day ($n=18$)	4.67	9.98	19.8	49.7	519	4120
Accuracy (arithm. mean value) [%]						
Day 1 ($n=6$)	90.1	98.9	100.0	101.2	102.6	106.2
Day 2 ($n=6$)	100.8	103.8	97.1	97.4	104.1	100.4
Day 3 ($n=6$)	89.3	98.5	100.6	99.5	104.4	102.5
Inter-day ($n=18$)	93.4	99.8	99.2	99.3	103.7	103.0
Precision (arithm. mean value) [%]						
Day 1 ($n=6$)	11.5	9.7	1.9	2.0	2.8	6.1
Day 2 ($n=6$)	12.5	7.7	6.1	7.9	7.0	1.1
Day 3 ($n=6$)	13.7	2.8	3.1	2.7	0.9	1.9
Inter-day ($n=18$)	13.2	7.5	4.1	4.9	4.2	4.3

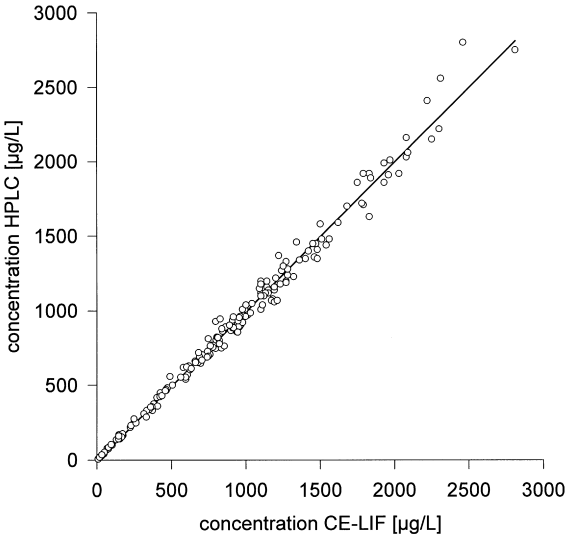


Fig. 8. Correlation of concentrations of moxifloxacin in plasma obtained with CE–LIF and HPLC (r^2=0.9897, slope=1.00, intercept=−8.86, n=216).

4.2. Comparison with HPLC

The CE–LIF assay for moxifloxacin in plasma and microdialysate showed the same reproducibility as HPLC. Formal validation experiments according to international guidelines proved that the assay is suitable for use in clinical studies. LOQ was comparable to HPLC. CE–LIF showed a wider working range than HPLC (2.5–5000 µg/l versus 2.5–1000 µg/l), which may be important for bioanalytical assays to save time/costs and to avoid errors by subdiluting. Analysis time and total run time were equivalent in the presented assay for routine use.

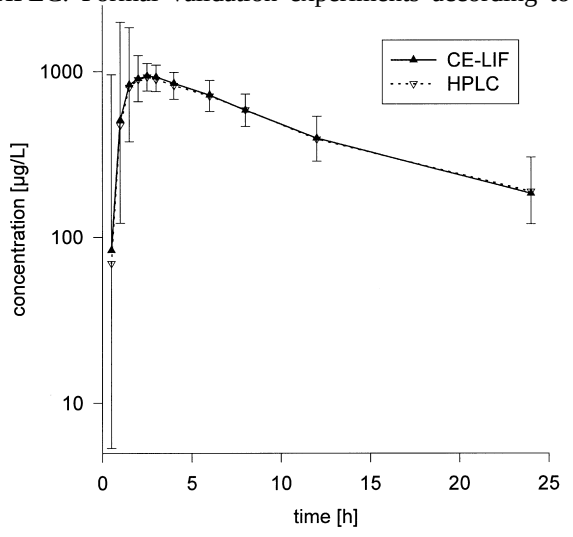


Fig. 9. Plasma concentration time profiles of moxifloxacin receiving a 200 mg oral dose of moxifloxacin determined by HPLC and CE–LIF (geo. mean/standard deviation of eight male volunteers; top standard deviation are for CE–LIF and down standard deviation are for HPLC).

CE–LIF can be an excellent alternative to HPLC for native fluorescing analytes in bioanalytical assays. At the moment this technique is limited to molecules that show native fluorescence around the wavelengths of the HeCd-lasers (=325 nm) and Ar-ion laser (=488 nm). Other wavelengths are accessible, but the required instruments are too expensive to be an alternative to HPLC at the moment. Although moxifloxacin is excited at 325 nm, which is far away from its five-fold higher maximum (λ=296 nm, Fig. 2), a LOQ of 2.5 µg/l can be achieved in plasma even after precipitation (=diluting 1+3). As this work has shown, it is not mandatory that the analyte is excited at its maximum. Therefore CE–LIF can be applicable to many other fluorophores with absorption around 325 nm. Further investigations to establish this method routinely also for other analytes, especially quinolones, are ongoing and look promising.

4.3. High sample throughput

Analysis times of less than 1 min could be achieved in this work after manual injection using a 7/27 cm capillary. Parallel analysis in 12 or many more capillaries is possible [17,18]. CE–LIF has a high potential to increase productivity with bigger autosamplers (preferably microtiter plates), parallel analysis and short effective length of capillaries. These optimizations have not been combined with LIF-detection at UV-wavelengths yet. Using the CE–LIF assay for moxifloxacin, a fast one step sample pretreatment could be combined with adequate instrumentation to achieve a high sample throughput.

5. Conclusion

A routine CE–LIF assay to measure samples in clinical studies was established successfully. This

well-controlled method generated high quality data with acceptable levels of precision, accuracy, working range and LOQ. CE–LIF was preferred to HPLC when only small sample volumes (<100 μl) were accessible. Therefore the method is suitable for situations with minimal matrix volumes: e. g. pediatrics, patients at risk, animal-, microdialysis- and tissue-kinetic studies. It would have been used more often also for normal sample volumes (1 ml) if a bigger autosampler had been available in combination with UV–LIF detection.

The potential to increase sample throughput is higher as for HPLC. CE has the possibility of working in parallel analysis modes while HPLC is restricted to sequential runs.

CE–LIF is being increasingly viewed as an alternative for, and complement to HPLC in bioanalytical assays. It could be shown that the analytical performance of CE–LIF is equivalent to HPLC and routine use of CE–LIF in clinical bioanalytics is possible.

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