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Capillary electrophoresis with laser-induced fluorescence detection, an adequate alternative to high-performance liquid chromatography, for the determination of ciprofloxacin and its metabolite desethyleneciprofloxacin in human plasma

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Abstract

A method to determine plasma concentrations of ciprofloxacin and its metabolite desethyleneciprofloxacin (M1) by CE with HeCd laser-induced fluorescence detection is described. Following precipitation of proteins and centrifugation supernatant is injected hydrodynamically (10 s, 0.5 p.s.i.) into the capillary. Overall analysis time for the quantification of both analytes was 7 min. The total amount of plasma needed for multiple injections ($n>5$) was 10–20 μl. Data on accuracy and precision are presented. The assay performance is compared to the specifications of a validated HPLC method, which is routinely used for the quantification of ciprofloxacin and M1 in body fluids. Both methods showed comparable accuracy and precision for both analytes throughout the whole working range (inter-day precision <9%; inter-day accuracy 96–110%). The limit of quantification (LOQ) of 20 μg/l (M1 10 μg/l) for the CE procedure was slightly higher than for the HPLC method, where 10 μg/l (M1 2.5 μg/l) was determined. However, application of the methods to human plasma samples derived from a clinical study proved that comparable results are obtained and that the sensitivity of the HPCE method was sufficient to fully describe typical plasma concentration time profiles of ciprofloxacin and its metabolite M1. Both the adequate sensitivity and the required smaller sample volume compared to HPLC indicate that the method is feasible for clinical studies where sample amounts are limited, e.g., studies to investigate pharmacokinetics in pediatric patients. Preclinical studies form another possible application of this technique.

Keywords: Ciprofloxacin; Desethyleneciprofloxacin

1. Introduction

Capillary electrophoresis (CE) has become a very useful tool for pharmaceutical analysis because of its high resolution, speed and the extremely small sample volume required [1]. There are many applications for quality control of pharmaceuticals and their formulations [2]. However, the use of CE in the bioanalysis of drugs and their metabolites is restricted due to the low concentration sensitivity of this technique. Hence there are only a few applications for the quantification of drugs in body fluids

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[3–5]. The use of laser-induced fluorescence detection (LIF) not only improves on the limit of quantification (LOQ) for analytes with native fluorescence but also allows the analysis of biological matrices with enhanced selectivity [6–9].

Ciprofloxacin (Fig. 1), a fluoroquinolone carboxylic acid, is an antibiotic with extended antibacterial spectrum including grampositive and gramnegative bacteria and *Pseudomonas aeruginosa*. The drug shows good fluorescence under acidic conditions after excitation at 325 nm and can easily be analyzed with CE-HeCd–LIF. The pharmacokinetics and metabolism have been investigated in depth using HPLC methods. However, most of these methods need large plasma volumes (0.5–5 ml) for the extraction of the drug [10,11].

Although ciprofloxacin has not yet been licensed for use in children, it is one alternative for the therapy of infections and their exacerbations for young cystic fibrosis patients [12]. One consequence of this restricted use of the drug in pediatric medicine is the paucity of relevant pharmacokinetic data. Because metabolism and pharmacokinetics in children can considerably differ from those of adults further investigations are needed to derive dosing guidelines.

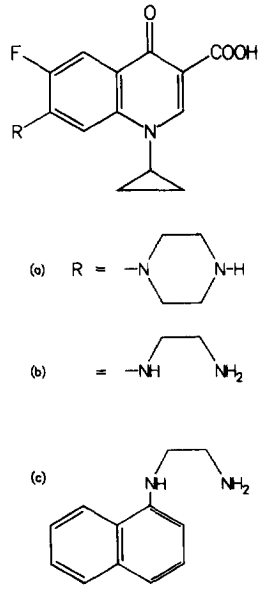

Fig. 1. Structure of ciprofloxacin (a), the desethylenemetabolite M1 (b) and the internal standard NED (c).

2. Experimental

2.1. Chemicals

Ciprofloxacin and M1 were used as certified reference compounds (Bayer, Wuppertal, Germany) for quantitative analysis.

N-(1-Naphthyl) ethylenediamine·di-HCl (NED) was purchased from Fluka Chemica, acetonitrile (HPLC-grade) and boric acid (99.5%) from Riedel de Haen. Ortho-phosphoric acid (<85%) and sodium hydroxide were obtained from E. Merck (Darmstadt, Germany), triethylamine from Sigma and tetrabutylammonium bromide >99% (TBA) from Fluka Chemica.

Water was purified using the Milli Q system (Millipore Waters, Eschborn, Germany).

2.2. Sample pretreatment for CE

Plasma was prediluted 9:1 with water. In preexperiments the influence of sample composition was investigated. Plasma samples (study and calibration) without the predilution step although correlating with the HPLC data of the same samples showed a systematic deviation (Fig. 2: black dots, without predilution). Addition of 1 part of water to 9 parts of

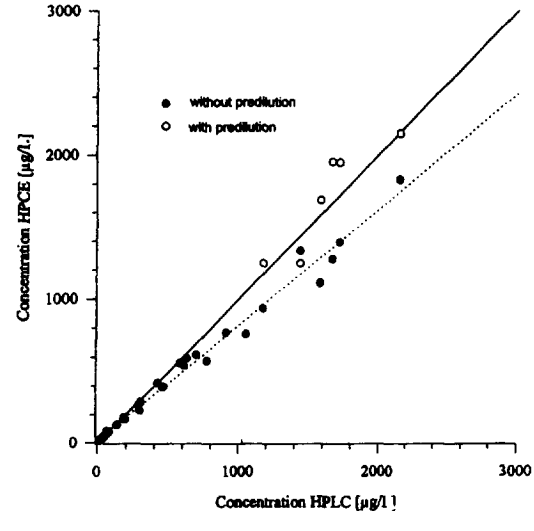

Fig. 2. Correlation of HPLC vs. CE results for ciprofloxacin concentrations in plasma from Phase I studies (dots and circles differ in the amount of proteins).

plasma led after corresponding calculation to the 1:1 correlation (Fig. 2: circles, with predilution).

40 μl of this pretreated solution (or less) were diluted 1:5 with a mixture of acetonitrile–0.1 *M* H_3PO_4 (9:1) containing the internal standard NED (0.2 μg/ml). After vortex mixing the suspension was centrifuged for 10 min at 3500 *g* to separate the precipitated proteins. The supernatant was injected into the capillary.

2.3. Sample pretreatment for HPLC

200 μl of plasma were diluted 1:1 with 0.1 *M* H_3PO_4. The solution was vortex mixed and centrifuged for 10 min at 3500 *g*. The supernatant was injected into the liquid chromatograph.

2.4. Electrophoresis equipment and operating conditions

A P/ACE Model 5010 (Beckman Instruments, Munich, Germany) with a fused-silica capillary was used for electrophoresis. Detection was performed using a HeCd-laser, Model 3074-20M (Omnichrome, Chino, CA, USA) with excitation wavelength of 325 nm (20 mW). Emission was measured at 450 nm using an interference filter. Data was collected with use of software Gold 8.10 (Beckman, Munich, Germany). The capillary had a total length of 37 cm (30 cm to detector) and an inner diameter of 50 μm. The outer diameter was 360 μm.

Separation was carried out in a 1:1 mixture of 0.1 *M* H_3PO_4 and 0.1 *M* H_3BO_3. The pH was adjusted with triethylamine to 2.3. The applied voltage was 28 kV and the temperature was maintained at 20°C.

Injection was performed hydrodynamically for 10 s at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). After each run the capillary was rinsed with 0.1 *M* NaOH and running buffer for 1 min.

2.5. HPLC equipment and operating conditions

A Hewlett Packard 1090M (Hewlett-Packard, Waldbronn, Germany) with a ternary gradient system, autoinjector and autosampler was used for chromatography. Detection was performed with a HP 1046A fluorescence detector (Hewlett-Packard). Excitation wavelength was 277 nm, emission was measured at 455 nm. To perform reverse phase chromatography a Nucleosil 100 RP18, 5 μm (250× 4.6 mm) from Muder and Wochele (Berlin, Germany) protected by a guard column (17×4.4 mm) containing the same stationary phase was used. The mobile phase consisted of acetonitrile and an aqueous solution of 0.05 mol/l TBA and 0.025 mol/l *ortho*-phosphoric acid adjusted to pH 2 with 0.1 mol/l sodium hydroxide solution. After separation of the analytes under isocratic conditions (11% acetonitrile for 6.2 min, flow-rate 1 ml/min) a step gradient was used to clean the column from residual components and eventually precipitated plasma proteins (50% acetonitrile for 3.5 min, flow-rate 1.5 ml/min) followed by reequilibration of the stationary phase (11% acetonitrile for 7 min, flow-rate 1 ml/min). The column oven temperature was maintained at 50°C using a column oven.

2.6. Calibration for CE

Calibration samples were obtained by spiking 9 parts of blank plasma with 1 part of an aqueous solution of ciprofloxacin and M1 to yield seven concentrations in the range of 20–2000 μg/l for ciprofloxacin and 10–200 μg/l for M1. Quality control samples were prepared in the same way at three levels for both analytes covering the same range of concentration. All samples were processed further as described in Section 2.2. beginning with dilution with the acetonitrile–H_3PO_4 mixture containing NED as internal standard and analysed in one sequence.

Calibration curves according to the internal standard method were obtained by plotting concentration vs. peak-area ratios. Linear regression with a $1/y^2$ weighting factor resulted in an optimal calibration function (Concalc software, Institute of Clinical Pharmacology, Bayer, Wuppertal). Other regression functions tested did not lead to a better curve fit or smaller residuals.

2.7. Calibration for HPLC

Calibration samples were obtained by spiking aliquots into blank plasma to yield seven concentrations in the range of 10–2000 μg/l for ciprofloxacin and 2.5–150 μg/l for M1. All samples were

processed further as described in Section 2.3 and analysed in one sequence. Calibration curves were obtained according to the CE method.

2.8. Assay validation for CE

Six spiked samples at concentrations of 20, 50, 100, 500, 1000 and 2000 μg/l for ciprofloxacin and 10, 20, 50, 100, 150 and 200 μg/l for the metabolite were analysed within one day to assess intra-day variability. The procedure was repeated on three days to investigate inter-day precision and accuracy. Absence of matrix interferences was confirmed by analysis of blank plasma.

2.9. Assay validation for HPLC

Five spiked samples at concentrations of 5, 10, 25, 250 and 1500 μg/l for ciprofloxacin and 2.5, 10, 25, 75 and 150 μg/l for the metabolite were analysed within one day to assess intra-day variability. The procedure was repeated on three days to investigate inter-day precision and accuracy. Absence of matrix interferences was confirmed by analysis of blank plasma.

2.10. Application

Both analytes were determined in plasma samples taken from a clinical pharmacological study. Three healthy male volunteers between 22 and 34 years received one 500 mg ciprofloxacin tablet with breakfast. Blood samples were obtained up to 24 h after administration. Within 30 min after sampling plasma was prepared by centrifugation and stored at −20°C until analysis.

3. Results

3.1. General aspects

Ciprofloxacin, its metabolite M1 and the internal standard (I.S.) were readily separated within 5 min from plasma constituents employing CE in untreated fused-silica capillaries with HeCd laser-induced fluorescence detection. The gain in sensitivity in comparison to UV detection at 280 nm was about two orders of magnitude under acidic run conditions. Interference from matrix components was negligible. Therefore plasma was simply diluted, the precipitate was removed and the supernatant was injected. Fig. 3 shows a typical electropherogram and Fig. 4 a chromatogram for blank plasma and plasma spiked with both analytes.

3.2. Validation

The method was validated in a concentration range of 20–2000 μg/l for ciprofloxacin and 10–200 μg/l for the metabolite. Calibration curves were obtained by plotting concentration vs. peak-area ratio (CE) and concentration vs. peak-area (HPLC), respectively. The calibration was $1/y^2$ weighted. The results of a formal validation experiment with CE are presented in Table 1, those obtained for ciprofloxacin with the HPLC method in Table 2. With CE inter-day precision for ciprofloxacin was <9% throughout the entire working range. Accuracy was also satisfactory (97–106%). Based on these results the limit of quantification (LOQ) was 20 μg/l for ciprofloxacin and 10 μg/l for M1. The LOQ of the HPLC method were 10 μg/l and 2.5 μg/l and the data on precision

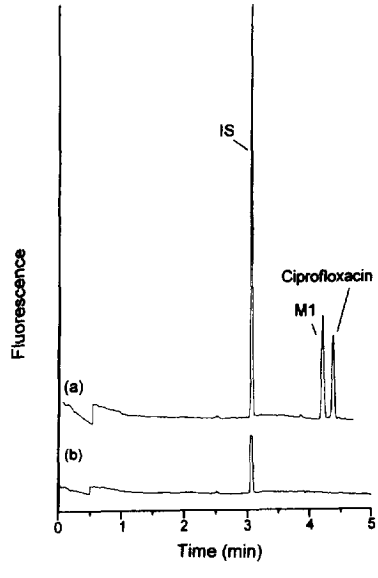

Fig. 3. Electropherogram of (a) human plasma spiked with ciprofloxacin (250 μg/ml), M1 (75 μg/ml) and internal standard (I.S.), (b) blank plasma spiked with I.S..

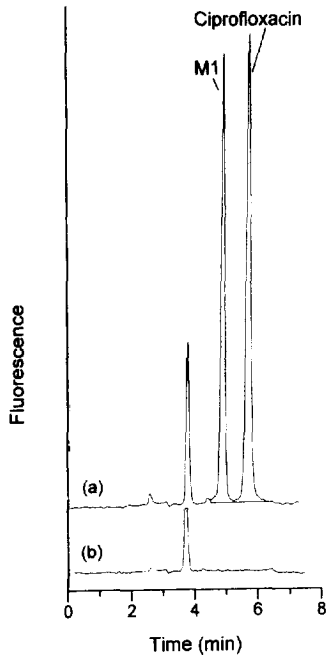

Fig. 4. Chromatogram of (a) human plasma spiked with ciprofloxacin and M1, (b) blank plasma.

(<6%) and accuracy (98–102%) for ciprofloxacin deviate less compared to the CE results.

3.3. Application

The method was applied to samples from a clinical study and the results were comparable to those of the HPLC-analysis. A comparison of the concentration–time profiles after HPLC- and CE-analysis of the same samples is given in Fig. 5.

4. Discussion

The use of laser-induced fluorescence detection in CE makes this technique an alternative to HPLC analysis with comparable concentration sensitivity in case of native fluorescent analytes like ciprofloxacin. Both methods lead to comparable results for the quantification of this drug and one of its metabolites from human plasma. Regarding the validation results the HPCE method fulfils the requirements but the HPLC shows better reproducibility.

Table 1
Intra-day and inter-day precision and accuracy of the determination of ciprofloxacin/M1 in human plasma with CE–LIF analysis

	Nominal concentration (μg/l) ciprofloxacin/M1					
	20/10	50/20	100/50	500/100	1000/150	2000/200
Concentration found (μg/l) (arithm. mean value)						
Day 1 (n=6)	21.24/10.88	50.43/19.51	101.5/52.00	501.3/106.3	966.4/153.9	2110.0/224.7
Day 2 (n=6)	20.70/10.04	52.46/18.95	106.9/49.47	489.4/95.5	952.0/141.3	2089.4/209.6
Day 3 (n=6)	19.21/9.86	52.32/19.66	103.2/51.58	522.0/104.6	1018.3/158.7	2105.9/222.1
Inter-day (n=18)	20.38/10.26	51.74/19.37	103.9/51.02	504.2/102.1	978.8/151.3	2101.8/218.8
Accuracy (%) (arithm. mean value)						
Day 1 (n=6)	106.2/108.8	100.9/97.5	101.5/104.0	100.3/106.3	96.6/102.6	105.5/112.3
Day 2 (n=6)	103.5/100.4	104.9/94.8	106.9/98.9	97.9/95.5	95.2/94.2	104.5/104.8
Day 3 (n=6)	96.0/98.6	104.6/98.3	103.2/103.2	104.4/104.6	101.8/105.8	105.3/111.0
Inter-day (n=18)	101.9/102.6	103.5/96.9	103.9/102.0	100.9/102.1	97.7/100.9	105.1/109.4
Precision (%) (arithm. mean value)						
Day 1 (n=6)	7.4/6.3	4.4/5.1	4.3/5.3	4.9/9.6	4.3/6.9	2.4/2.6
Day 2 (n=6)	9.4/7.5	7.0/5.7	1.8/2.5	1.3/2.3	4.3/3.1	4.2/3.0
Day 3 (n=6)	7.7/5.0	9.2/7.2	5.6/3.4	3.4/4.1	4.8/4.9	5.5/4.2
Inter-day (n=18)	8.9/7.5	7.0/6.0	4.5/4.3	4.3/7.7	5.2/7.0	4.0/4.4

Table 2
Intra-day and inter-day precision and accuracy of the determination of ciprofloxacin in human plasma with HPLC analysis

	Nominal concentration (μg/l) ciprofloxacin				
	5.11	10.19	25.44	253.1	1519
Concentration found (μg/l) (arithm. mean value)					
Day 1 (n=6)	4.91	10.66	25.11	247.9	1506
Day 2 (n=6)	5.79	10.16	25.23	248.0	1501
Day 3 (n=6)	4.82	9.92	25.77	250.7	1528
Inter-day (n=18)	5.17	10.25	25.37	248.9	1512
Accuracy (%) (arithm. mean value)					
Day 1 (n=6)	96.2	104.6	98.7	97.9	99.1
Day 2 (n=6)	113.4	99.7	99.2	98.0	98.8
Day 3 (n=6)	94.3	97.3	101.3	99.0	100.5
Inter-day (n=18)	101.3	100.5	99.7	98.3	99.5
Precision (%) (arithm. mean value)					
Day 1 (n=6)	7.3	5.5	2.5	0.7	0.9
Day 2 (n=6)	8.6	2.9	2.6	2.3	2.1
Day 3 (n=6)	8.8	5.5	2.4	1.3	0.7
Inter-day (n=18)	11.7	5.5	2.6	1.6	1.5

Sample pretreatment for HPCE analysis includes one more step of dilution compared to that for HPLC. This step is necessary to yield samples with equal amount of proteins compared to the spiked calibration samples. The amount of proteins obviously influences the quantification, probably caused by co-precipitation of both analytes or differences in sample viscosity. The loss in sensitivity caused by this dilution can partly be compensated by the stacking effect in the acetonitrile–water mixture.

Comparing the running time of both methods the CE analysis is 3-fold faster than HPLC but the small capacity of the autosampler is the limiting factor for the use of CE in routine analysis.

However, for preclinical studies and especially for studies with children where only small sample

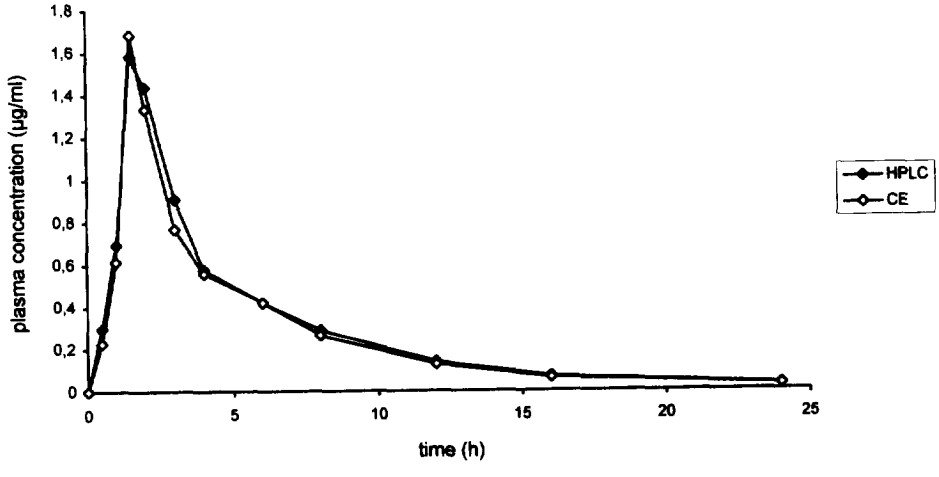

Fig. 5. Comparison of plasma concentrations of ciprofloxacin obtained by HPLC and CE.

volumes can be obtained, CE analysis is a good alternative to HPLC, because it can be performed several times from a sample volume of only 10 μl.

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