Enantioselective Assay of Chloroquine and Its Main Metabolite Deethyl Chloroquine in Human Plasma by Capillary Electrophoresis*

Dirk Müller and **Gottfried Blaschke**

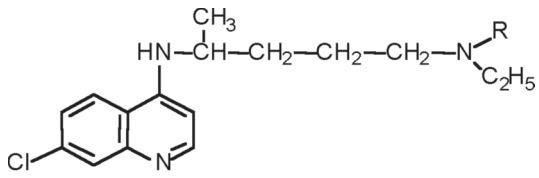
Institute of Pharmaceutical Chemistry, University of Münster, Hittorfstr. 58-62, D-48149 Münster, Germany.

Abstract

A sensitive assay for the determination of chloroquine (Clq) and its pharmacologically active metabolite deethyl chloroquine in plasma by capillary electrophoresis (CE) is developed. Plasma levels of drug and metabolite are measured using HeCd laser-induced fluorescence (LIF) detection over a range of three orders of magnitude from 2 to 1000 ng/mL after liquid–liquid extraction. A limit of detection of 0.5 ng/mL is achieved. Validation of the method yields intra- and interday precision data within the limits of 10% (20% at limit of quantitation) and intra- and interday accuracy data greater than 6% throughout the whole working range. The method is applied for the drug monitoring of patients treated with Clq. Based upon this assay, two enantioselective CE–LIF methods for Clq and its main metabolite are developed. Mixtures of substituted γ-cyclodextrins are used as chiral selectors. A baseline separation of the enantiomers of both analytes in one run is achieved in less than 11 min (method A) and less than 9 min (method B), respectively. Hydroxychloroquine is used as the internal standard for both methods.

Introduction

Chloroquine (Clq) is the most important drug in the treatment as well as the prophylaxis of malaria for over forty years. According to the World Health Organization, approximately 270 million people are infected and approximately 1 million die each year of complications from malaria. Recent studies have tried to understand the development of resistances observed in some strains of *Plasmodium falciparum* (1–3). Other therapeutic indications for Clq are rheumatoid arthritis, amoebic dysentery, and lupus erythematodes (4). The drug monitoring of Clq and its pharmacologically active main metabolite deethyl chloroquine (DeClq) is important for optimal clinical use, because a narrow therapeutic range and a high interindividual variability of plasma levels (5,6) can result in toxic blood concentrations (Figure 1).



	R
Clq	CH_2CH_3
DeClq	H
OHClq	CH_2CH_2OH

Figure 1. Structures of Clq, DeClq, and the IS.

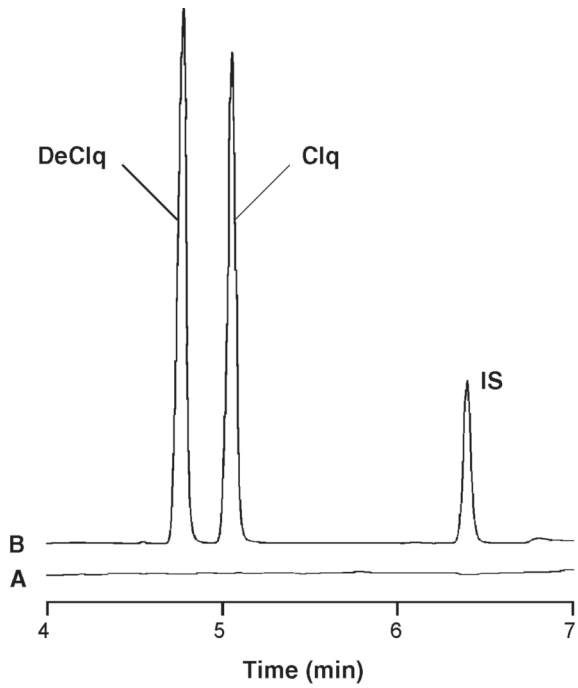


Figure 2. Electropherograms of blank plasma (A) and plasma spiked with 500 ng/mL Clq, 500 ng/mL DeClq, and 270 ng/mL IS (B).

* Dedicated to Prof. Richard Neidlein (Heidelberg) on the occasion of his 70th birthday.

† Author to whom correspondence should be addressed: e-mail blaschg@uni-muenster.de.

Capillary electrophoresis (CE) with its small sample volumes required, its short analysis times, and its use of small volumes of aqueous buffers offers an attractive alternative to high-performance liquid chromatography (HPLC) in bioanalysis. The CE method described in this study has been validated according to accepted guidelines (7,8) and applied to measure levels of Clq and DeClq in a patient's plasma during therapy. Hydroxychloroquine (OHClq) was used as the internal standard (IS). The usually poor detection limit (a problem in bioanalysis by CE) was overcome by the sensitive and selective laser-induced fluorescence (LIF) detection. In combination with offline and online analyte concentration procedures, the limit of detection (LOD) could be lowered to 0.5 ng/mL for both Clq and DeClq, which is lower than in established HPLC methods with fluorescence detection (9,10). A wide working range of 2 to 1000 ng/mL (for both analytes as well as small sample volumes (200 µL) of plasma that is needed for the assay) is a further advantage of the CE method.

Table I. Intra- and Interday Precision and Accuracy of Clq and DeClq

Clq	**Spiked plasma concentrations of Clq (ng/mL)**					
	1049.4	209.9	21.0	10.5	5.25	2.10
Concentration found (mean value) (ng/mL)						
Day 1 (*n* = 6)	1030.7	211.7	20.3	10.3	5.18	2.00
Day 2 (*n* = 6)	1050.1	209.0	20.2	10.5	5.22	2.19
Day 3 (*n* = 6)	1034.0	212.9	20.4	10.2	5.12	2.16
Interday (*n* = 18)	1038.3	211.2	20.3	10.3	5.17	2.12
Precision (mean value, %)						
Day 1 (*n* = 6)	3.9	2.6	2.9	4.1	8.55	10.57
Day 2 (*n* = 6)	2.1	2.3	3.5	7.6	6.18	10.56
Day 3 (*n* = 6)	2.6	3.4	3.3	7.6	6.70	9.45
Interday (*n* = 18)	2.9	2.7	3.1	6.3	6.83	10.44
Accuracy (mean value, %)						
Day 1 (*n* = 6)	–1.8	0.9	–3.2	–1.9	–1.31	–4.93
Day 2 (*n* = 6)	0.1	–0.4	–3.9	–0.2	–0.60	4.18
Day 3 (*n* = 6)	–1.5	1.5	–2.9	–2.7	–2.50	3.05
Interday (*n* = 18)	–1.1	0.6	–3.3	–1.6	–1.47	0.77
DeClq	**Spiked plasma concentrations of DeClq (ng/mL)**					
	1019.1	203.8	20.4	10.2	5.09	2.04
Concentration found (mean value, ng/mL)						
Day 1 (*n* = 6)	1036.7	202.8	20.8	10.0	5.10	2.04
Day 2 (*n* = 6)	1008.0	203.6	20.5	10.1	5.07	1.90
Day 3 (*n* = 6)	1022.5	206.0	21.3	11.0	4.81	1.93
Interday (*n* = 18)	1022.4	204.1	20.9	10.3	4.99	1.95
Precision (mean value, %)						
Day 1 (*n* = 6)	2.2	2.4	2.2	3.4	6.96	9.59
Day 2 (*n* = 6)	2.6	2.6	2.7	4.4	6.29	9.86
Day 3 (*n* = 6)	2.4	2.3	1.7	1.2	9.58	12.96
Interday (*n* = 18)	2.5	2.4	2.7	5.6	7.74	10.69
Accuracy (mean value, %)						
Day 1 (*n* = 6)	1.7	–0.5	1.9	–2.4	0.28	–0.03
Day 2 (*n* = 6)	–1.1	–0.1	0.7	–1.2	–0.38	–6.66
Day 3 (*n* = 6)	0.3	1.1	4.7	8.1	–5.75	–5.88
Interday (*n* = 18)	0.3	0.2	2.4	1.5	–1.95	–4.19

Clq and DeClq show a stereoselective metabolism (11,12). In CE, enantioselective separations can be performed using chiral selectors as additives that are simply dissolved in the running buffer. The two chiral CE methods presented in this study achieved the separation of Clq and DeClq enantiomers in one run with a mixture of different substituted γ-cyclodextrins (CDs) as chiral selectors. In HPLC, the enantioselective analysis of both analytes require the achiral separation of Clq and DeClq prior to the chiral separation of the single enantiomers (13). Chiral method A was applied for the analysis of spiked and real plasma samples. Similar to the achiral CE method, OHClq could also be used as the IS.

Experimental

Chemicals and materials

Clq diphosphate was purchased from Sigma (St. Louis, MO). DeClq and OHClq sulfate were obtained from Sterling Winthrop (Rensselaer, NY).

Disodium tetraborate decahydrate, sodium hydroxide, hydrochloric acid, and ethyl acetate (HPLC-grade) were purchased from E. Merck (Darmstadt, Germany). Spermine tetrahydrochloride was purchased from Sigma (St. Louis, MO). Water was purified with the Milli-Q system (Millipore Waters, Eschborn, Germany). Hydroxypropyl (HP)-γ-CD was supplied as a kind gift from Wacker Chemie (Munich, Germany). Monosulfated γ-CD was a kind gift from Prof. Gyula Vigh (A&M University, Texas). Carboxymethyl (CM)-γ-CD was purchased from Cyclolab (Budapest, Hungary). All reagents were used as purchased.

Instrumentation

The achiral analysis was performed on a Beckman P/ACE 5010 instrument with an LIF detector from Beckman (Munich, Germany). An Omnichrome HeCd-laser (3074-20M, Laser 2000, Wessling, Germany) with a 20-mW and 325-nm excitation wavelength was connected by an optical fiber (Omnichrome POS FDS - A 1/2, Laser 2000, Wessling, Germany) to the detector. For the detection, a 405-nm emission filter was used. The collected data were integrated and analyzed using the software Gold 8.10 from Beckman (Munich, Germany). To prevent the sample from evaporating, the instrument was equipped with a cooled sample tray and cryogenic vials. A Haake D8 water bath from Haake (Karlsruhe, Germany) was used to keep the sample tray temperature at 13°C. The liquid cooling of the capillary was set to 16°C. The analysis was carried out in an air-conditioned room at 20°C. Capillaries with an effective length of 30 cm (total length 37 cm) and a 50-µm i.d. with an extended light path (bubble cell) were obtained from Hewlett-Packard (Waldbronn, Germany).

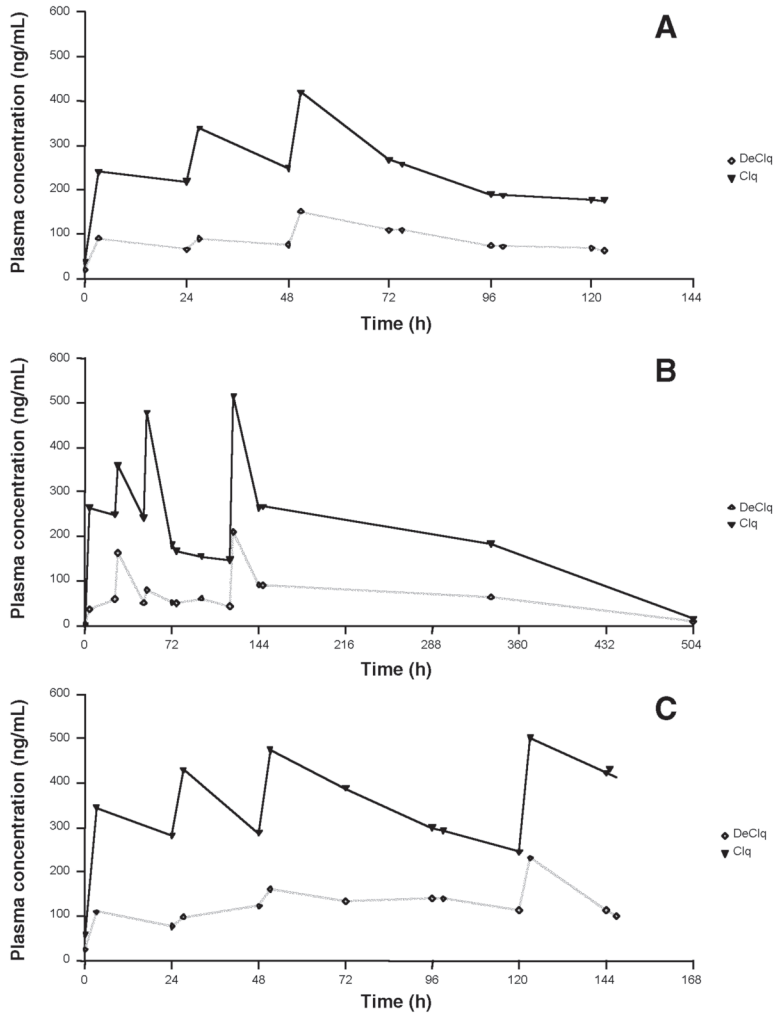


Figure 3. Concentration-time profiles of patients 1 (A), 2 (B), and 3 (C) after repeated oral intake of 600 mg Clq.

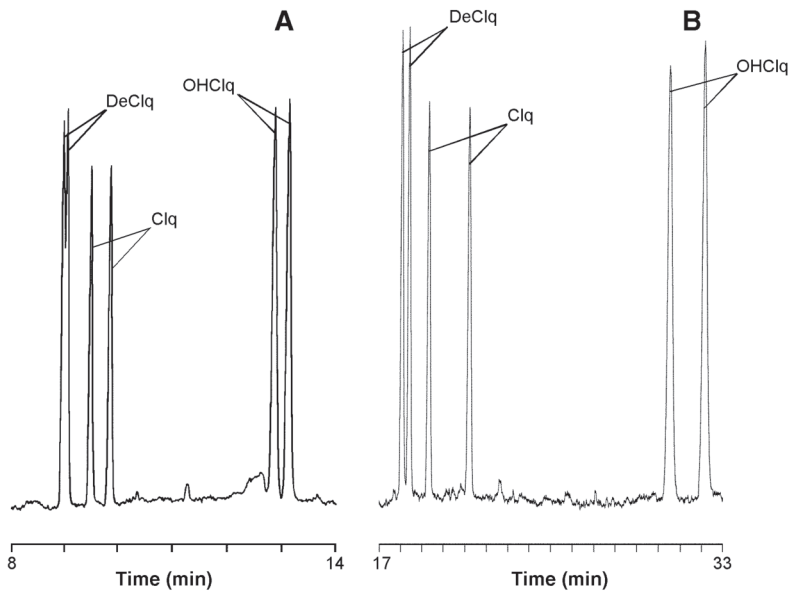


Figure 4. Electropherograms of Clq, DeClq, and OHClq without (A) and with (B) 4 mg/mL spermine added to the anodic buffer (100mM disodium tetraborate decahydrate, pH 10, 15 mg/mL HP-γ-CD).

The chiral analysis was performed on a Beckman P/ACE 2100 instrument with an LIF detector as described previously. The liquid cooling of the capillary was set to 20°C. The analysis was carried out at room temperature (20–25°C).

Buffer preparation

The running buffer used for the achiral determination in plasma was 100mM disodium tetraborate adjusted to pH 10.25 by 5M sodium hydroxide. The alkaline buffer was chosen because of the pH-dependent fluorescence emission of Clq with a maximum pH level of approximately 10. A 100mM disodium tetraborate buffer adjusted to pH 12.5 by 5M sodium hydroxide was used for extraction.

For chiral separation, a 100mM disodium tetraborate buffer adjusted to pH 9.65 by 5M sodium hydroxide was used. CDs used as chiral selectors and spermine tetrahydrochloride used for the modification of the electro-osmotic flow (EOF) were added to this buffer.

The anodic buffer in method A contained 105 µg/mL spermine tetrahydrochloride and 10.0 mg/mL HP-γ-CD. CM-γ-CD was dissolved in the cathodic buffer to a concentration of 8.0 mg/mL.

For method B, the anodic buffer contained 64 µg/mL spermine tetrahydrochloride and 12.5 mg/mL HP-γ-CD. The concentration of monosulfated γ-CD in the cathodic buffer was 5.0 mg/mL.

Prior to usage, all buffers prepared were degassed for 5 min in an ultrasonic bath.

Sample preparation

For validation, nine parts of drug-free plasma were spiked with one part of a solution of Clq and DeClq in 10mM HCl in the desired concentration. The spiked plasma samples and solutions were stored at –20°C and under exclusion of light. To a 200-µL aliquot of spiked plasma, 40 µL of OHClq solution (1.62 µg/mL in 10mM HCl) was added as the IS. After 10 s of vortexing, 200 µL of the extraction buffer was added. The alkalized plasma was vortexed for another 10 s and extracted with 1200 µL ethyl acetate during 30 s of vortexing, followed by centrifugation for 10 min at 4000 rpm. A volume of 1100 µL of the supernatant ethyl acetate layer was evaporated to dryness using a vacuum centrifuge. The residue was reconstituted in 20 µL of 10mM HCl and injected into the CE system. For chiral analysis, the sample volume was reduced to 10 µL. Therefore, the extraction could be carried out with half the volume of plasma and extraction reagents. To prevent the adsorption of Clq to glass, the samples were stored and the complete extraction was carried out in polypropylene containers (Eppendorf caps) (14).

Assay conditions

In the achiral CE method, the capillary was rinsed with 0.5M NaOH (90 s) and the running buffer (90 s) prior to each sample injection. A zone of purified water was hydrodynamically injected for 1 s with 0.5 psi, followed by electrokinetic sample injection with 10 kV for 7 s. During separation, the voltage was set to 13 kV in normal polarity mode, which resulted in a field strength of 351 V/cm and a current of approximately 130 µA. Migration times were 4.9 min for DeClq, 5.1 min for Clq, and 6.6 min for the IS, which accounts for a total analysis time of 10 min per sample.

In the chiral CE methods A and B, the capillary was rinsed according to the following scheme to prevent an inner coating of the capillary with spermine and CDs, which led to unreproducible migration times of 30 s for purified water, 60 s for 0.5M HCl, 30 s for purified water, 60 s for 0.5M NaOH, and 90 s for the anodic buffer. A zone of purified water was hydrodynamically injected for 1 s with 0.5 psi, followed by electrokinetic sample injection with 10 kV for 10 s. In method A, the separation was carried out at 12 kV, which lead to a field strength of 324 V/cm and a current of approximately 90 µA. Migration times were 9.2 min and 11.0 min for the two Clq enantiomers, 9.6 min and 10.6 min for the two DeClq enantiomers, and 12.4 min and 13.7 min for the two OHClq enantiomers. In method B, the voltage had to be set to 15 kV (resulting in a field strength of 405 kV and a current of approximately 100 µA) in order to achieve baseline separation of the enantiomers. The migration times were reduced to 7.5 min and 7.8 min for the DeClq enantiomers, 8.3 min and 8.7 min for the Clq enantiomers, and 9.3 min and 9.5 min for the OHClq enantiomers.

Results and Discussion

Achiral determination

Recovery

Because the electrokinetic injection depended on the conductivity of the sample, plasma extracts could not be directly compared with the aqueous solutions of the analytes because of the matrix effect. Therefore, the plasma extracts were compared with spiked plasma samples. Human plasma samples were prepared as described previously, with the exception that the residue was redissolved in 30 µL instead of 20 µL of the 10mM buffer. The reference samples were spiked with the analyte and IS. After extraction, the residue was reconstituted with 10 µL of the Clq and DeClq solution and 20 µL of the IS solution. Recovery rates for a concentration of 500 ng/mL were 91.4% ± 2.3% for Clq and 90.9% ± 2.4% for DeClq.

The recovery rate for the IS was measured to be 89.8% ± 0.4% (Figure 2).

Calibration and validation

Calibration curves for Clq and DeClq were obtained by measuring spiked plasma samples of eight concentration levels within the range of 2 to 1000 ng/mL. The time-corrected peak areas of Clq, DeClq, and the IS were correlated against the sample concentrations of the analytes by the IS method. Linear regression showed correlation coefficients higher than 0.99 for both analytes. Precision and accuracy of the method were determined for six concentration levels (2, 5, 10, 20, 200, and 1000 ng/mL) with six spiked plasma samples at each level. Using two different calibration curves from 2 to 20 ng/mL and 20 to 1000 ng/mL precision for Clq and DeClq with a standard deviation above 10% for both analytes was acceptable. Accuracy stayed within ± 10% and ± 20% at the limit of quantitation (LOQ) for Clq and DeClq. All plasma samples and calibration samples were assayed during one day for the calculation of intraday variability. The validation experiment was repeated on two more days to also assess the interday variability. Precision and accuracy are presented in Table I.

The LOD was found to be 0.5 ng/mL in plasma for Clq and DeClq when a signal-to-noise ratio of 1:3 as the LOD was considered. The absence of any matrix interferences was confirmed by the analysis of drug-free plasma (Figure 2A).

Application

The CE–LIF method was used to monitor Clq and DeClq plasma levels in three patients treated with a high-dosage level of Clq. Each patient received 600 mg of Clq diphosphate orally at day 1, 2, and 3 always at the same time. Patient 2 and 3 received another 600-mg dose at day 5. Plasma samples were taken twice each day from every patient for six days, sample 1 was taken right before the intake of the dose and sample 2 three hours later to monitor peak concentrations in plasma (15). Two more samples were collected on day 14 and 21 from the second patient. The plasma concentrations versus time profiles for the patients are presented in Figure 3.

The developed method proved to be suitable to monitor the plasma concentrations of all three high-dosage patients. The wide concentration range covered the peak concentrations of greater than 500 ng/mL Clq without subdiluting the samples. The LOQ of

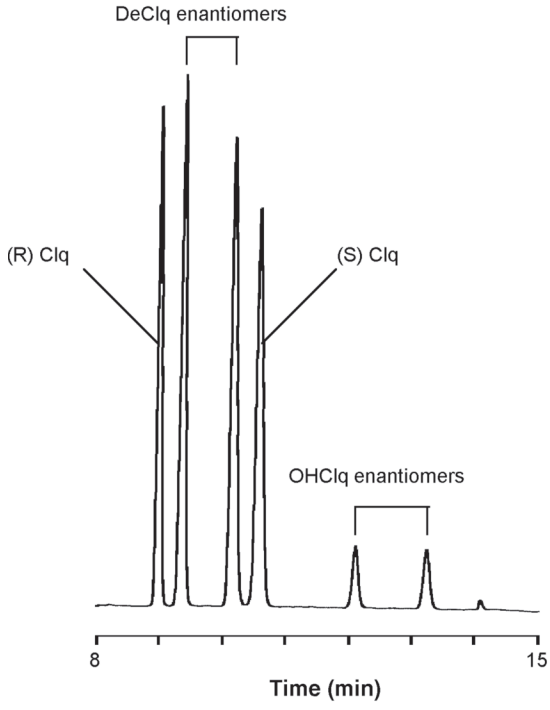


Figure 5. Electropherogram of plasma spiked with 600 ng/mL Clq, 600 ng/mL DeClq, and 200 ng/mL OHClq (chiral method A).

2 ng/mL for Clq and DeClq allowed for the monitoring of the long terminal elimination phase. All three patients had already been treated with Clq prior to this study, which resulted in low Clq and DeClq plasma levels at $t = 0$. To calculate qualified basic pharmacokinetic parameters, plasma samples should have been collected more often to provide better concentration-time profiles, and also a prolonged wash-out phase should have been kept.

Enantioselective separation

The enantioselective separation of both Clq and DeClq enantiomers in one run was achieved by the modification of the EOF and a combination of two different chiral selectors dissolved in the running buffer. HP-γ-CD acted as a chiral selector in both methods. Because this CD was neutral, it migrated with the cathodic EOF while the two analytes and the IS migrated as cations in front of the EOF. A complete baseline separation (with HP-γ-CD as the only chiral selector) could not be achieved. Only the combination with spermine tetrahydrochloride for the dynamic coating of the inner capillary wall provided an almost baseline separation of all analytes (Figure 4).

The ammonium groups of spermine tetrahydrochloride were attracted to the silanol groups of the capillary wall, which reduced the mobility of the EOF according to their concentration in the running buffer (16). This improved the separation but prolonged the migration times to over 30 min. A baseline separation in a short analysis time could be realized by the combination of HP-γ-CD, spermine tetrahydrochloride, and a negatively charged CD as a second chiral selector. At alkaline buffer pH, CM-γ-CD was negatively charged and migrated in opposite direction to the analytes towards the anode. This counter-flow principle provided excellent chiral separations. The optimization of the concentrations of spermine, HP-γ-CD, CM-γ-CD, and buffer pH resulted in a baseline separation of Clq, DeClq, and OHClq enantiomers in 14 min. Spiked and real plasma samples were analyzed with chiral method A. The electropherograms are presented in Figures 5 and 6. The configuration of Clq peaks in the electropherograms was assigned by spiking the analytes with Clq enantiomers of a known configuration.

The different peak areas for the enantiomers of Clq and DeClq in Figure 6 indicate the stereoselective metabolism of Clq. However, the configuration could not be assigned because enantiomers of DeClq were not available for us. A further improvement regarding analysis time could be achieved with chiral method B, in which the negatively charged CM-γ-CD is replaced by monosulfated γ-CD. The separation time could be reduced to less than 10 min and the migration order of the enantiomers changed to the two DeClq enantiomers, which migrate faster than the two Clq enantiomers (Figure 7).

The chiral determination method described for Clq and DeClq is an offline combination of achiral and chiral HPLC assays (13). Both chiral CE methods A and B were able to separate the Clq and DeClq enantiomers in one run in 14 min and 10 min, respectively, with OHClq as the IS for quantitation.

Conclusion

An achiral CE–LIF assay can be established successfully to measure concentrations of Clq and its active metabolite DeClq in plasma samples. The common problem of low sensitivity in CE was solved by using sensitive LIF detection. A capillary with an extended light path (bubble cell) and an optimal fluorescence emission of Clq at pH 10.25 further enhanced sensitivity. Sample stacking (injection of a water zone prior to sample injection) and the electrokinetic sample injection lowered the LOD to 0.5 ng/mL. These techniques allowed for the quantitation of Clq and DeClq in the concentration range from 2 to 1000 ng/mL.

Furthermore, two chiral CE–LIF methods could be developed for the enantioselective separation of Clq and DeClq with short

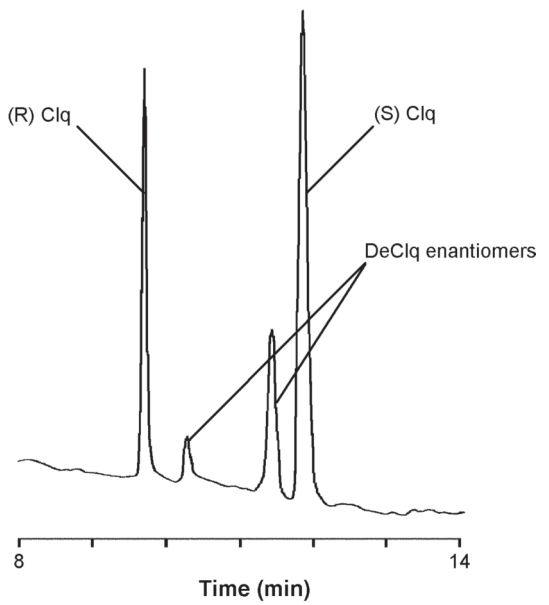


Figure 6. Electropherogram of a plasma sample taken 3 h after oral intake of 600 mg racemic Clq diphosphate (chiral method A).

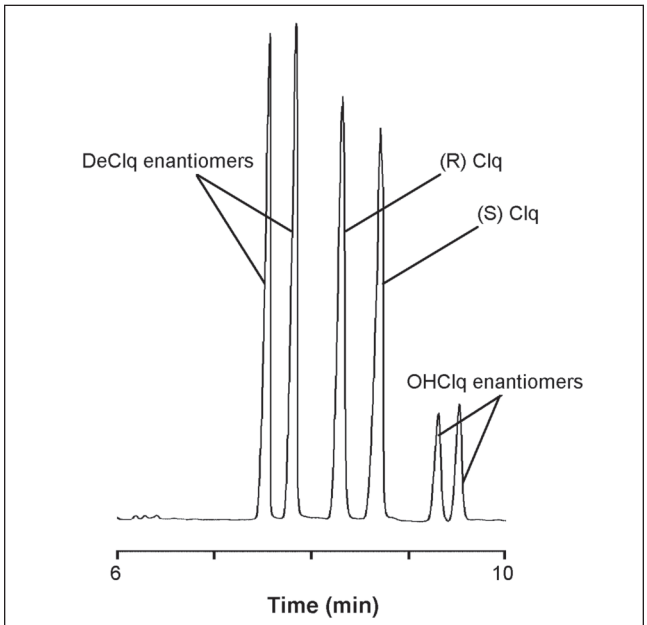


Figure 7. Electropherogram of plasma spiked with 600 ng/mL Clq, 600 ng/mL DeClq, and 200 ng/mL OHClq (chiral method B).

run times of 14 and 10 min. In both methods, OHClq was used as the IS for quantitation.

Acknowledgments

The authors wish to thank Dr. G. Ahr (Bayer AG, Wuppertal, Germany) and Dr. J.G. Möller (Bayer AG, Wuppertal, Germany) for providing the CE–LIF instrument for validation, and specifically Dr. J.G. Möller for the scientific discussions and support during validation. The authors would also like to express their thanks to Prof. Dr. G. Vigh (A&T University, Texas) and Wacker Chemie for kindly supplying the CD derivatives.

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Manuscript accepted August 4, 2000.