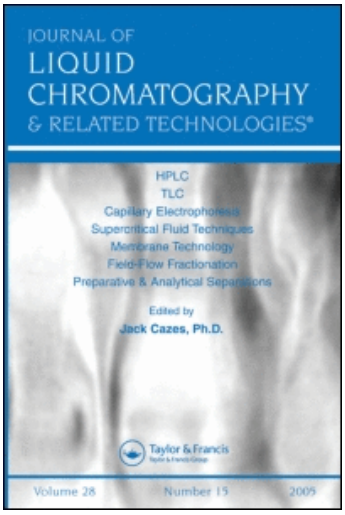

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Chloroquine and Desethylchloroquine in Biological Samples Using Perfusion Chromatography and Fluorescence Detection

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To cite this Article Augustijns, P.(1997) 'Determination of Chloroquine and Desethylchloroquine in Biological Samples Using Perfusion Chromatography and Fluorescence Detection', Journal of Liquid Chromatography & Related Technologies, 20: 7, 1103 — 1113
To link to this Article: DOI: 10.1080/10826079708010962
URL: http://dx.doi.org/10.1080/10826079708010962

J. LIQ. CHROM. & REL. TECHNOL., 20(7), 1103–1113 (1997)

DETERMINATION OF CHLOROQUINE AND DESETHYLCHLOROQUINE IN BIOLOGICAL SAMPLES USING PERFUSION CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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ABSTRACT

A high-speed reversed phase HPLC method for the determination of chloroquine and desethylchloroquine in biological samples is presented. Perfusion chromatography, based on the use of flow-through particles made of cross-linked polystyrene/divinylbenzene, allowed to increase the flow-rate of the mobile phase to 4 mL/min, resulting in base-line separation of *bis*-desethylchloroquine, desethylchloroquine, chloroquine, and the internal standard in less than 3 minutes. As the column material withstands prolonged exposure to high pH conditions, the pH of the mobile phase could be adjusted to 9.5, enabling direct determination of chloroquine and desethylchloroquine by fluorescence detection after elution; therefore, no post-column alkalinization to obtain the pH of optimal fluorescence was required. Interday as well as intraday reproducibility for chloroquine concentrations ranging from 31.3 to 500 ng/mL in plasma was lower than 7%. The applicability of the method is illustrated by measuring blood concentrations of chloroquine and desethylchloroquine in blood during 1 week after oral intake of 500 mg chloroquine.

INTRODUCTION

Since its introduction forty years ago, chloroquine has been the most important drug in the treatment and prophylaxis of malaria. Although the emergence of chloroquine-resistant strains of *Plasmodium falciparum* and *Plasmodium vivax* has reduced its efficiency, chloroquine remains an important antimalarial drug in tropical Africa and Asia.[1,2] Chloroquine is also being used in the treatment of rheumatoid arthritis and systemic lupus erythematosus.[3,4] The determination of chloroquine concentrations in biological samples is important for several reasons, such as the assessment of patient compliance, the determination of pharmacokinetic data and the prevention of toxic blood concentrations after prolonged use, especially in the case of the treatment of rheumatoid arthritis. Reliable analysis methods are also required for quality control of chloroquine preparations. Spectrophotometry,[3] fluorimetry,[6] GLC,[7-9] HPLC,[10-18] and ELISA[19] have all been used for the determination of chloroquine in biological samples. Recently, a CE method has been described for the bioanalysis of antimalarial drugs.[20] In HPLC, fluorescence detection offers superior sensitivity to UV detection, but requires normal phase chromatography[16,17] or reversed phase chromatography combined with post-column alkalinization.[18] Packing consisting of cross-linked polystyrene/divinylbenzene withstands prolonged exposure to high pH conditions, so that pH can be adjusted directly to the pH of optimal fluorescence, making post-column alkalinization redundant. In addition, the presence of flow-through pores of 6000-8000 Å in the polymeric packing of perfusion chromatography columns allows the mobile phase to flow through the packing material, while diffusion is possible in smaller pores (800-1500 Å) that are connected to them.[21] The presence of these pores allows to increase the flow-rate of the mobile phase, resulting in high sample turn-over. The high-speed HPLC analysis described in this paper is an improvement of a method developed earlier in our laboratory.[18]

MATERIALS AND METHODS

Reagents

Chloroquine diphosphate and diethylamine were obtained from Sigma (St. Louis, USA); desethylchloroquine and 6,8-dichloro-4-(1-methyl-4-diethyl-aminobutylamino)quinoline were kindly supplied by Sterling Drug Inc. (Rensselaer, N.Y.), and *bis*-desethylchloroquine by Rhône-Poulenc; acetonitrile and tetrahydro-furan were obtained from BDH (Poole, England)

and Sigma-Aldrich (Gillingham, England), respectively. Dichloromethane was from Rathburn (Hojbjerg, Denmark) and di-sodium tetraborate from UCB (Leuven, Belgium). All reagents were used as received.

Chromatographic Conditions

The HPLC system consisted of a Waters (Millford, Mass.) 600E System Controller, a Rheodyne injector equipped with a 100 μL loop, and a fluorescence detector (Model SFM 25, Kontron; Ex. 331 nm, Em. 381 nm). Chromatograms were recorded and processed using the Maxima 820 computer program. The column used was a 4.6-mm diameter x 100 mm length (1.7 mL column bed volume) POROS ® R/H (PerSeptive Biosystems, Cambridge, MA). The packing consisted of 10 μm flow-through particles of cross-linked polystyrene/divinylbenzene. Mobile phase A consisted of an aqueous buffer containing 20 mM di-sodium tertraborate, 0.1 % diethylamine and 20% acetonitrile (pH adjusted to 9.5 with 2N NaOH). Mobile phase B consisted of 100% tetrahydrofuran. Separation was obtained using the following gradient: after an initial stage of 0.3 min in mobile phase A, mobile phase B was increased to 4% and, at 0.6 min, to 10% by a step gradient; from 0.6 to 3 min, mobile phase B was increased by a linear gradient from 10 to 15%; to flush the column, mobile phase B was further increased over 0.5 min to 40 %, followed by an isocratic stage of 1 min and a return to initial conditions over 0.5 min; column re-equilibration in mobile phase A was obtained in 1 min. The flow rate of the mobile phase amounted to 4 mL/min. Both mobile phases were filtered and degassed continuously by He sparging. The injection volume amounted to 100 μL. The column was kept at a temperature of 60 °C.

Sample Preparation

Samples were prepared according to the method described earlier.[18] In short, plasma (150 μL) or blood (75 μL) samples were alkalinized with 0.5 mL NaOH 2N after addition of 100 μL internal standard solution (6,8-dichloro-4-(1-methyl-4-diethylaminobutyl-amino)quinoline, 1 μg/mL in HCl 0.2 N). After extraction with 7.0 mL dichloromethane and centrifugation (4000 rpm, 10 min), the water layer was discarded and the organic layer evaporated to dryness under a gentle stream of air. The residue was dissolved in an aliquot of mobile phase A and 100 μL was injected into the HPLC system. To prevent adsorption of chloroquine on glass, 0.1% diethylamine was added to the NaOH solution used for alkalinization.[22]

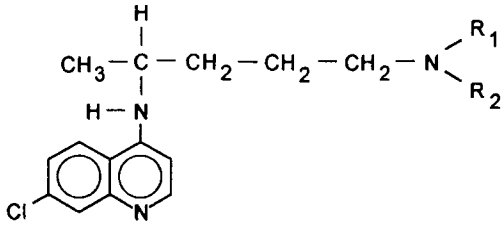

Figure 1. Structure of Chloroquine (R_1 = R_2 = C_2H_5) and its main metabolites desethylchloroquine (R_1 = C_2H_5; R_2 = H) and *bis*-desethylchloroquine (R_1= R_2 = H).

Calibration Plot

Stock solutions of desethylchloroquine, chloroquine and the internal standard were prepared at a concentration of 1 mg/mL in MeOH/H_2O (50/50). Drug-free plasma was spiked with aliquots of the stock solutions of desethylchloroquine and chloroquine, and handled similarly as the samples. A calibration graph was constructed in which concentration was plotted against the peak/area-ratio. All concentrations calculated are the average of two separate determinations.

RESULTS AND DISCUSSION

For the development of an HPLC bioanalysis of chloroquine and its main metabolite (Figure 1), fluorescence detection is preferred to UV detection because of superior sensitivity. Fluorescence in aqueous solution shows a maximum at a light alkaline pH,[6] which is illustrated in Figure 2. While chloroquine and desethylchloroquine show an obvious maximum between pH 8.5 and 10, the pH dependence of the fluorescence of the internal standard was smoother. A pH of 9.5 was chosen for all further experiments. Unfortunately, conventional reversed phase columns only withstand mobile phases with a maximum pH of 8.0, thus requiring post-column alkalinization for optimal fluorescence. To enable direct determination of chloroquine and its metabolites, packing material of cross-linked polystyrene/divinylbenzene was used. This material withstands prolonged exposure to extreme pH conditions, so that the pH of the mobile phase could be directly adjusted to the pH of optimal detection (pH 9.5). The through-pores present in the packing material allowed to increase the speed of the mobile phase to 4 mL/min, without increasing the back-pressure, offering the additional advantage of rapid analysis.

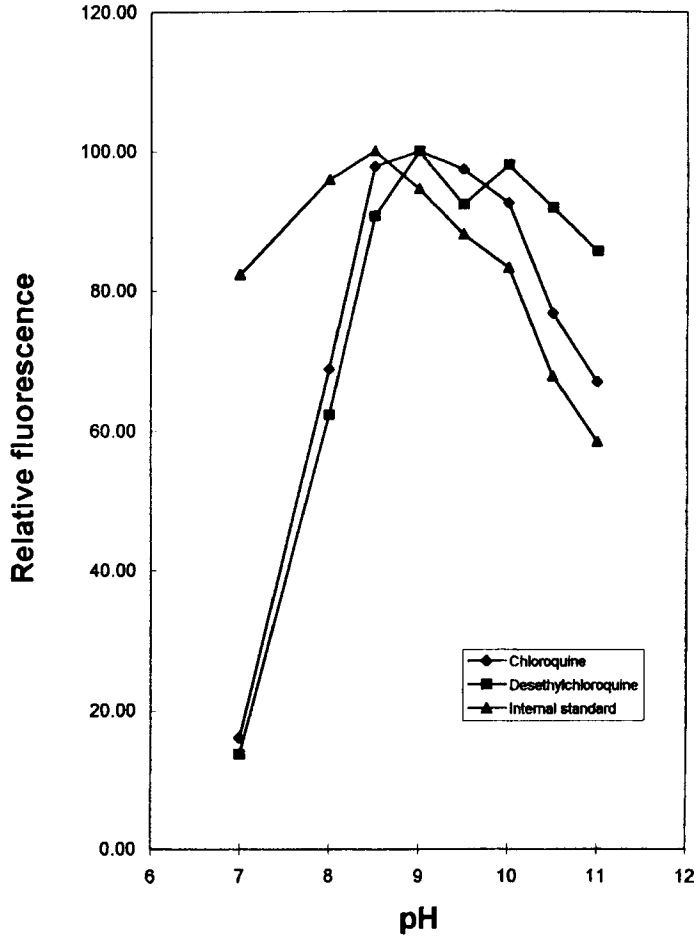

Figure 2. Relative fluorescence of chloroquine, desethylchloroquine and the internal standard as a function of pH (max. = 100%).

Using a gradient, base-line separation of chloroquine, desethyl-chloroquine, bisdesethylchloroquine and the internal standard could be obtained in about 2.5 min; re-equilibration in mobile phase A took 1 min, resulting in a total analysis time of less than 4 min. A wash-step up to 40% of mobile phase B could be included, as described in the materials and methods section. This short analysis time allowed to increase the sample turn-over and to decrease the apparatus occupation time. Typical chromatograms obtained by the final method are shown in Figure 3.

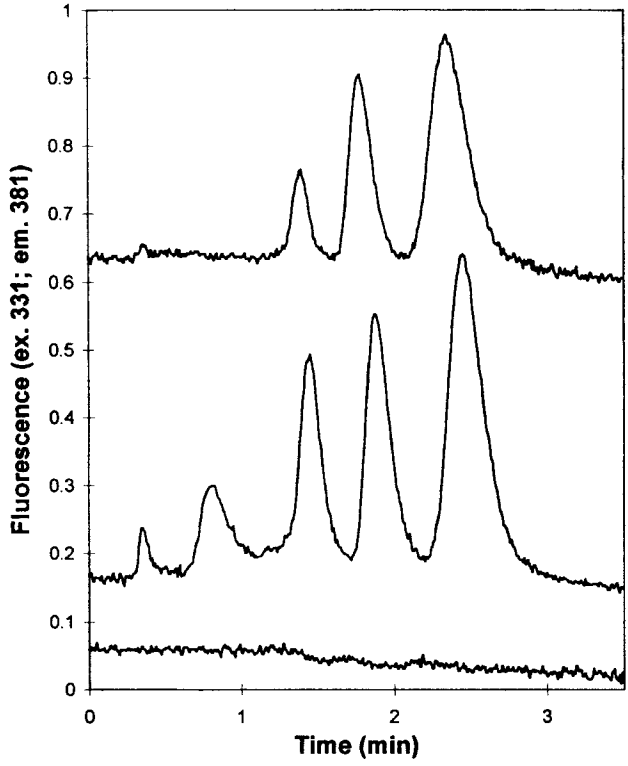

Figure 3. Chromatograms of a blank plasma sample (lower panel), a test solution containing *bis*-desethylchloroquine (rt = 0.8 min), desethylchloroquine (rt = 1.45 min), chloroquine (rt = 1.9 min), and the internal standard (rt = 2.45 min), and a sample prepared from plasma spiked with 31.3 ng/mL desethylchloroquine and 125 ng/mL chloroquine (upper panel).

The precision of the assay was assessed for chloroquine (31.3 - 500 ng/mL) and desethylchloroquine (15.6 -125 ng/mL) spiked in pooled human plasma, and is expressed as relative standard deviation in Table 1 (chloroquine) and Table 2 (desethylchloroquine). Calibration plots of chloroquine and desethylchloroquine showed good correlation ($r > 0.998$). From the chromatograms, a detection limit, which corresponded to a peak height of 3 times the noise of the baseline, was calculated as approximately 5 ng/mL for chloroquine and desethylchloroquine. As only 150 μL plasma or 75 μL blood was used in this study, increasing the sample volume will definitely improve the sensitivity of the assay.

Table 1

Reproducibility of Chloroquine Bioassay

Concentration Added (ng/mL)	Concentration Found (ng/mL)	RSD* (%)
Intraday Variation (n = 7)		
500	510	3.8
250	245	5.4
125	126	4.5
62.5	58.3	3.4
31.3	33.9	3.7
Interday Variation (n = 5)		
500	498	3.2
250	240	4.7
125	120	3.0
62.5	60.3	2.2
31.3	31.1	4.5

RSD = Relative Standard Deviation.

Table 2

Reproducibility of Desethylchloroquine Bioassay

Concentration Added (ng/mL)	Concentration Found (ng/mL)	RSD* (%)
Intraday Variation (n = 7)		
125	129	7.3
62.5	62.9	9.8
31.3	32.7	5.5
15.6	15.4	5.9
Interday Variation (n = 5)		
125	129	4.3
62.5	62.8	14
31.3	31.1	3.6
15.6	15.5	11

RSD = Relative Standard Deviation.

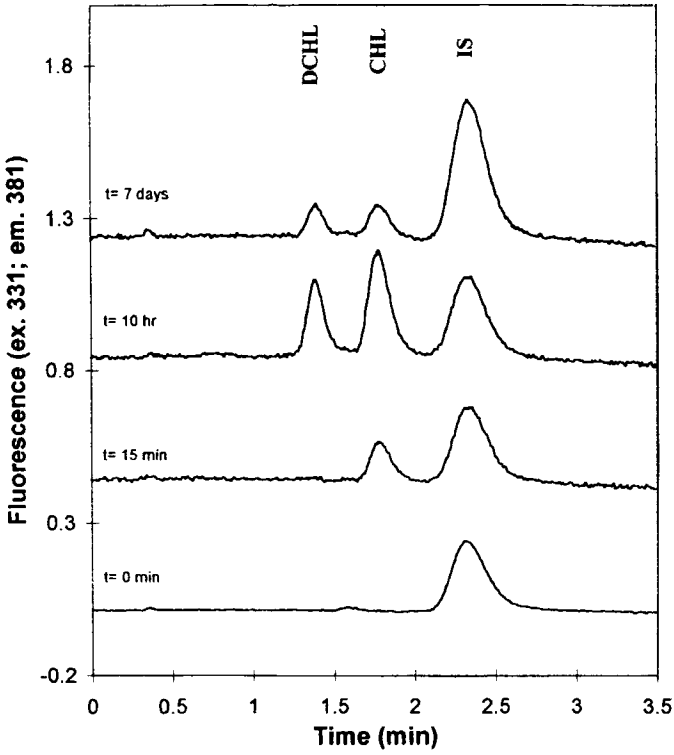

Figure 4. Chromatograms displaying the elimination and metabolism of chloroquine as a function of time after oral intake of 500 mg chloroquine at t=0 (DCHL = desethylchloroquine, CHL = chloroquine, IS = internal standard).

The applicability of the method is demonstrated by analyzing blood samples from a human subject, following an oral dose of 500 mg chloroquine. Figure 4 shows representative chromatograms at different time points; it can be seen that there is no substantial difference between chromatograms obtained from a blood sample and the chromatograms obtained from spiked plasma samples (Figure 3). As the extraction recovery from plasma and blood samples was reported to be in the same range,[18] total blood concentrations were calculated from a calibration plot made up in plasma. The use of total blood samples (75 μL) instead of plasma samples offers the advantage that concentrations are 2-5 times higher than those in plasma, due to accumulation in erythrocytes.[23] The concentration-time profile shown in Figure 5 illustrates that the detection limit mentioned is sufficient to calculate basic pharmacokinetic parameters.

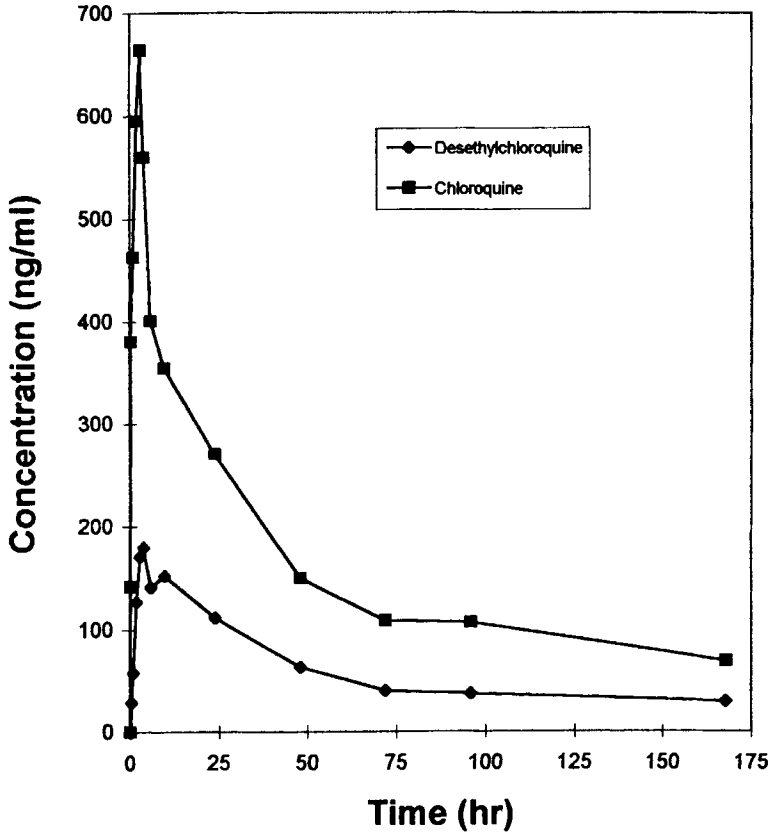

Figure 5. Concentration-time profile of chloroquine and desethylchloroquine during 1 week after oral intake of 500 mg chloroquine.

In summary, these results demonstrate that chloroquine and desethylchloroquine can be determined in biological samples by a high speed HPLC method based on the use of perfusion chromatography; the high pH possible on the cross-linked polystyrene/divinylbenzene packing opens the possibility to adjust the pH of the aqueous mobile phase to that of optimal fluorescence of chloroquine, enabling direct determination after elution from the column. The method can easily be adapted for quality control of chloroquine preparations.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek (NFWO). Ig. H. Herbots is acknowledged for technical assistance.

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Received August 18, 1996
Accepted September 30, 1996
Manuscript 4266