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Quantitative determination of chloroquine and desethylchloroquine in biological fluids by high performance thin layer chromatography

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Summary

A high performance thin layer chromatograpic (HPTLC) method for chloroquine (CQ) and desethylchloroquine (DCQ) determinations in plasma, erythrocytes and urine is described. Samples extracted by heptane from an alkaline water phase are separated on HPTLC silica gel plates, using toluene/diethylamine (9:1). The compounds are quantified by scanning fluorescent signals. The detection limit is 0.01 µmol/l for CQ and DCQ, with an extraction efficiency of 76±7%. Further simplification could make the method suitable for use in field surveys.

Key words: chloroquine; desethylchloroquine; plasma; urine; HPTLC.

Introduction

Determination of antimalarial drug concentrations in the blood or urine is necessary when it is essential to discover whether a patient has been taking antimalarials as instructed, and thus to ensure the correct interpretation of pharmacological field studies (Verdier et al., 1985). It is also important to determine the exact drug levels in the plasma and erythrocytes of patients with malarial infections, where the parasites show resistance in in vitro tests. This would facilitate the correlation of results from in vitro tests with in vivo drug concentrations (WHO, 1984; Smrkovski et al., 1985).

The Dill-Glazko eosin test (Lelijveld and Kortmann, 1970) is still widely used to determine the presence of chloroquine in urine, but it is neither sufficiently specific nor sensitive (Rombo et al., 1985). Essien and Afamefuna (1982) described a thin layer chromatographic method to separate chloroquine and its metabolites in urine and whole blood, but the method lacks sufficient sensitivity to determine quantitatively the chloroquine in plasma and erythrocytes.

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A specific and sensitive measurement of chloroquine (CQ) and its main metabolite, desethylchloroquine (DCQ), was made possible through the introduction of HPLC (Alvan et al., 1982; Bergqvist and Frisk-Holberg, 1980). The use of these techniques in field surveys is significantly restricted due to the need for an analytical laboratory. A simple method to determine the levels in the blood quickly and relatively easily would therefore be of considerable value.

We describe a quantitative thin layer chromatographic method, based on instrumentalized sample application and fluorescence detection, to determine chloroquine with a high specificity and sensitivity. Its possible simplification for use in field surveys is discussed.

Material and Methods

Chloroquine-free urine and blood samples were received from healthy volunteers. Blood stabilized by EDTA, heparin or citrate was centrifuged 1–3 h after collection, at 1000 *g* for 10–15 min (Bergqvist and Domeij-Nyberg, 1983). The buffy coat was removed by aspiration and the plasma and the erythrocytes were frozen at –20°C until assayed. Routinely 1 ml plasma, urine and/or 0.33 ml erythrocytes brought to 1 ml with distilled water and with different concentrations (10–200 ng/ml of an equal mixture of CQ [Sigma, St. Louis] and DCQ [kindly provided by Winthrop AG, Basel, Switzerland]) were used for the extraction of chloroquine and desethylchloroquine. The samples were diluted with 3 ml H_2O and made alkaline by adding 1 ml 5 N NaOH. The extraction was performed by adding 5 ml heptane/isoamyl alcohol (100:1.5 v/v) by shaking gently in 1 min intervals during 20 min. The layers were separated after standing for 10 min, or by centrifugation for 5 min at low speed in the case of the extraction from erythrocytes. Four ml of the heptane phase was transferred into a 5 ml glass reaction vial and evaporated to dryness under a stream of nitrogen on a Reacti-therm (Pierce Eurochemie, Holland) at 45°C. CQ and DCQ were taken up in 50 µl of 70% ethanol at 4°C with the Hamilton syringe of a Linomat III sample applicator (CAMAG, Switzerland). The solution was applied as a 0.5 cm long strip onto a HPTLC silica gel plate 60 (10×20 cm; Merck, Darmstadt), prewashed with toluene/diethylamine (9:1 v/v). CQ and DCQ were separated by chromatography in a glass tank using a mixture of toluene/diethylamine (9:1).

One hour after development the dried plate was evaluated in a CAMAG TLC Scanner (CAMAG, Switzerland). Fluorescence was measured by excitation with a mercury lamp at 313 nm and emission with a cut-off filter at 340 nm. Peak heights were used to calculate the concentrations of the different samples. The determination of the drug concentrations was done either graphically or by linear regression analysis of the peak heights. Peak identification was made by using the corresponding Rf-values (Rf = total distance moved by substance/distance of solvent front from origin).

Results

The extraction of the samples with heptane resulted in an overall extraction efficiency of 76±7% (Table 1). Chromatography of chloroquine and desethylchloroquine in toluene/diethylamine separated the two compounds with Rf values of 0.82 for CQ and 0.27 for DCQ (see also Fig. 1). The fluorescence method guaranteed a high sensitivity and specificity. The fluorescence quenching behind the CQ peak due to an unknown component in some plasma samples did not interfere with the determination of CQ concentrations (Fig. 1 C). The chromatograms of CQ and DCQ from urine and from erythrocytes are very similar and are not shown.

Table 1. Reproducibility of the chloroquine determination

Amount of chloroquine diphosphate used	Standard without extraction		Extracted sample	
	mean*	C.V.**	mean	C.V.
Reproducibility on one HPTLC plate				
16 ng	1.3 (8)	2.9%	1.0 (6)	15.0%
80 ng	6.3 (8)	1.4%	4.0 (7)	3.9%
Reproducibility between different HPTLC plates				
16 ng	1.3 (6)	8.6%	1.0 (7)	11.3%
80 ng	6.1 (6)	6.9%	4.7 (7)	8.7%

* mean of peakheight in cm; number of determinations in parenthesis
** C.V. = coefficient of variation

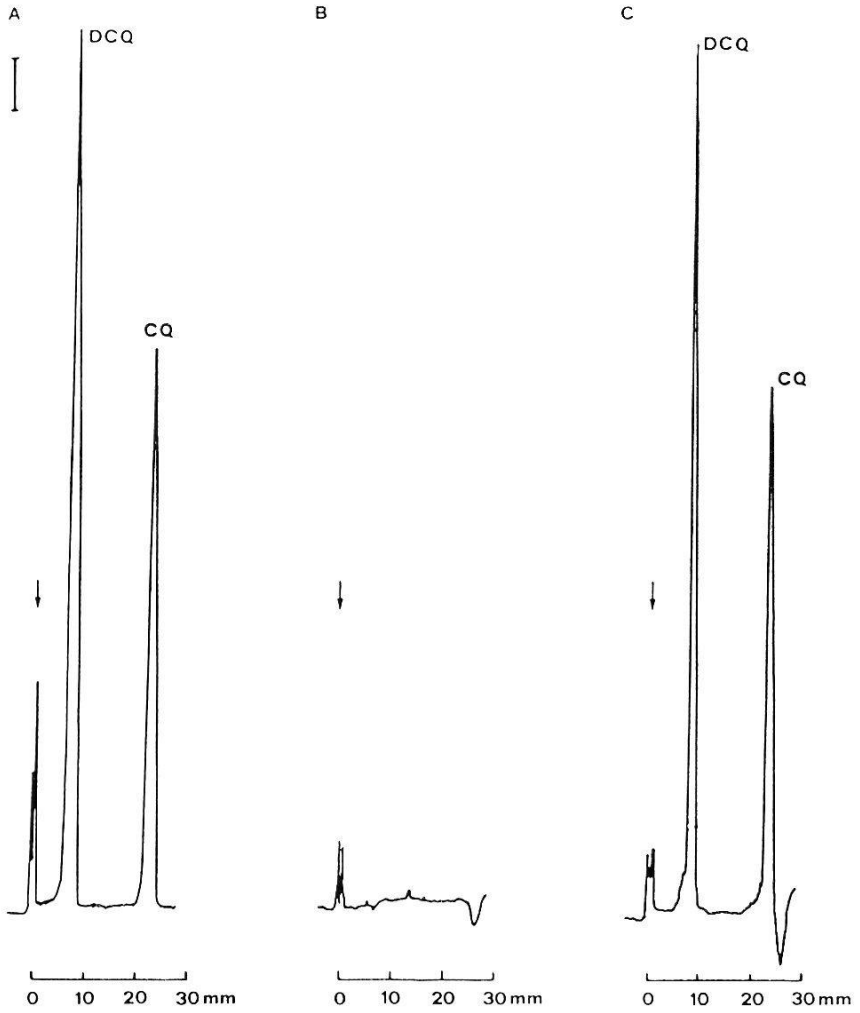

Fig. 1. Chromatograms of chloroquine and desethylchloroquine directly applied on HPTLC plates (160 ng each) (A), blank plasma (B), and a standard concentration (200 ng/ml) extracted from plasma. Arrows indicate points of application on HPTLC plates. Bar (A) indicated 1 cm peak height.

Through the use of diethylamine, a strongly basic compound, the fluorescence was increased, so that the minimal detectable quantity of 10 nml CQ/l could be determined. Immediately after chromatography a strong fluorescence

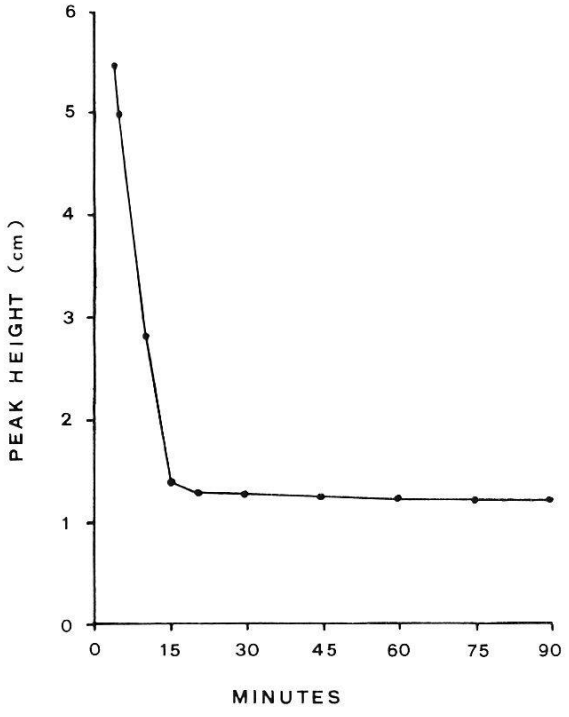

Fig. 2

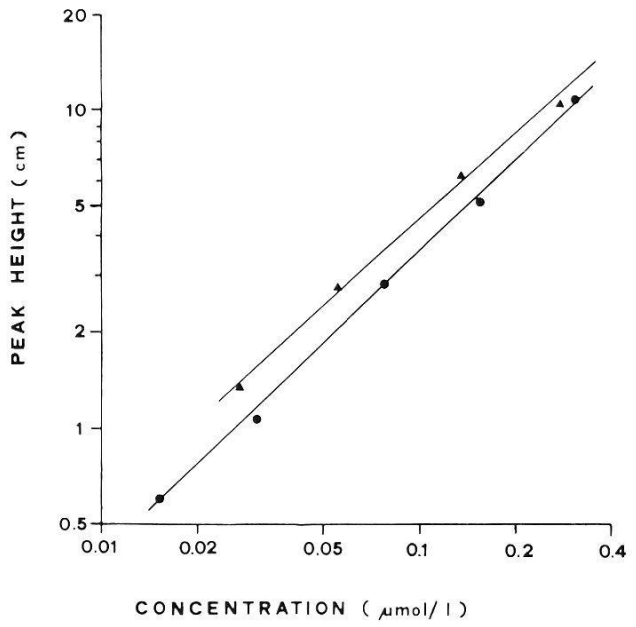

Fig. 3

Fig. 2. Decrease in fluorescence intensity after chromatography. 16 ng of chloroquine diphosphate were directly applied on the HPTLC plate and the fluorescence was measured after chromatography at the indicated time points.

Fig. 3. Calibration standard curves of CQ (●) and DCQ (▲) extracted from plasma.

could be detected, but the signal was not stable. One hour after removal of the plates from the chromatography tank the fluorescence of CQ and DCQ was stable enough to permit the scanning of a whole plate without any detectable change in the intensity of the fluorescence (Fig. 2). The relationship of the peak-height to the concentration was linear at least over the range 20–500 nmol/l with a correlation coefficient of $r = 0.995$ (Fig. 3). Desethylchloroquine always showed a stronger fluorescence (Fig. 3).

Discussion

The development of sensitive and specific techniques for the determination of chloroquine and its metabolites opened up the way for a renewed interest in the pharmacokinetic parameters of the 4-aminoquinolines (Gustafsson et al., 1983; Adelusi et al., 1982; Walker et al., 1983; Verdier et al., 1985). High performance liquid chromatography made it possible to study the distribution of chloroquine in the different blood compartments (Bergqvist et al., 1983). Most of the methods used are dependent on well-equipped laboratories; this makes studies related to field surveys and the continuous monitoring of drug levels difficult. The use of filter-papers on which blood from a finger-prick has been absorbed facilitates sample transport (Patchen et al., 1983). This method

only allows the determination of the total amount of chloroquine in the whole blood. No distinction is possible between the drug levels in the plasma and in the erythrocytes, both of which are the important parameters for the interpretation of the drug distribution in the blood.

Thin layer chromatography is a relatively simple and fast method for the determination of drugs. The conventional classical methods allowed a semiquantitative determination of the compounds separated and were used, for example, for a study of the distribution of chloroquine between maternal and child blood (Essien and Afamefuna, 1982). The compounds were detected by the use of Dragendorff's reagent. The Dragendorff reagent is not sensitive enough to work with small blood volumes and the method was not further developed.

The high performance thin layer chromatographic (HPTLC) method presented here allows the quantitative determination of CQ and DCQ using minimal amounts of blood or urine. The method was established with 1 ml samples of plasma and urine, but the actual amount needed to determine the minimal inhibitory concentrations (0.1μmol/1) (Bruce-Chwatt, 1981) in plasma is 100 μl. It is therefore possible to work with blood collected from finger pricks, an important aspect in field studies involving children (Burnier et al., 1984). Fifteen samples can be assayed at once on a 10×20 cm thin layer plate with a conventional glass tank; smaller plates can be used if fewer samples have to be analyzed. By using HPTLC linear chambers it is possible to increase the throughput to at least 30 samples in one run.

The method described is still dependent on a quantitative application instrument and a fluorescent detector. For field application these conditions limit its wide use, and should be replaced if possible. By using solid phase extraction methods the extraction procedure can be simplified (Good and Andrews, 1981). As a further alternative, if plasma and urine samples with high CQ concentration are expected, the samples could simply be deproteinised and immediately used for chromatography (Bergqvist and Frisk-Holmberg, 1980). Replacing the instrumentalized sample application by a simple capillary tube and using concentrating thin layer plates (which are commercially available) would still make it possible to obtain a good separation of chloroquine from its main metabolite without losing the accuracy of quantitative application. The detection by fluorescence is clearly superior due to its innate linearity, sensitivity and specificity, but appropriate scanning instruments are only available in large laboratories. However, for qualitative and semiquantitative evaluations a battery-powered fluorescent lamp would be sufficient to monitor the plates.

Several reagents exist to demonstrate nitrogen containing compounds, some of which, for example, have already been used for visualization of CQ (Essien and Afamefuna, 1982). It remains to be shown whether any of these reagents could replace fluorescence detection.

The HPTLC method described here has been successfully used to study the

influence of various types of breakfast on chloroquine levels (Lagrave et al., 1985) and to monitor the chloroquine levels in patients with chloroquine-resistant *P. falciparum* strains (Stahel et al., in preparation).

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