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DISTRIBUTION OF CHLOROQUINE AND ITS METABOLITE DESETHYL-CHLOROQUINE IN HUMAN BLOOD CELLS AND ITS IMPLICATION FOR THE QUANTITATIVE DETERMINATION OF THESE COMPOUNDS IN SERUM AND PLASMA

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SUMMARY

The amount of chloroquine and desethyl-chloroquine was determined in samples of total blood and in blood cell fractions from three normal subjects after one oral dose of 1000 mg of chloroquine diphosphate. About 70—85% of the total whole blood content of choroquine and its metabolite desethyl-chloroquine were recovered in blood cells isolated from whole blood, indicating that these compounds have a high cell/plasma concentration ratio. They were mainly present in thrombocytes and granulocytes.

A study of 40 patients taking chloroquine regularly as a treatment for rheumatoid arthritis showed significantly higher concentrations of chloroquine and desethyl-chloroquine in serum than in plasma. The concentration of chloroquine was about two times higher in serum than in plasma and for desethyl-chloroquine the concentration was about four times higher in serum than in plasma. These differences may be explained by a release of chloroquine and desethyl-chloroquine from thrombocytes during the coagulation of blood. The practical implication of the results is that the samples taken for chloroquine determination must be clearly identified as serum or plasma.

INTRODUCTION

In the measurement of drug concentration, binding of drugs to the cellular components of human blood has hitherto received little attention. Ehrnebo et al. [1] reported that 45—51% of the amount of pentazocine in whole blood is bound to blood cells. For chlorthalidone the amount in whole blood is ten times larger [2] than in plasma. At a given plasma concentration of tricyclic antidepressants, a six-fold interindividual variation in the erythrocyte levels was reported [3]. For digoxin the amount in erythrocytes is 2—6 times greater

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than in plasma [4], and the amount of carbamazepine in erythrocytes is 11—23% of the amount in plasma [5].

It has been shown that the time elapsed between the collection and centrifugation of blood significantly effects the plasma concentrations of gentamicin [6] and furosemide [7].

In evaluating the significance of drug concentration in blood for routine therapeutic drug monitoring it is therefore important to know whether the analytical determinations were carried out on whole blood, plasma or serum.

Some basic drugs such as chloroquine (CQ) [8] are accumulated in the organelles in thrombocytes. Thrombocytes have been shown to strongly bind another basic drug, imipramine [9].

Despite the widespread use of CQ in the prevention of malaria and in the treatment of rheumatoid arthritis, relatively little is known about its distribution in blood cells.

The aim of the present study was to measure the distribution of CQ and its metabolite desethyl-chloroquine (CQM) in blood cells, i.e. erythrocytes, granulocytes and thrombocytes, both in vivo and in vitro, and thereby evaluate some aspects of the sample handling before the assay of CQ and CQM.

EXPERIMENTAL

Blood samples and sample handling

Studies of the in vivo blood cell binding were performed on three healthy volunteers aged 35—46 years (two male and one female). They were given one oral dose of 1000 mg of chloroquine diphosphate. After 7 h, samples of antecubital venous blood were withdrawn into tubes containing EDTA.

Furthermore, EDTA-stabilized venous blood from two of these persons was used for experiments of the in vitro uptake of CQ and CQM in blood cells (about 7—8 months after the oral dose of 1000 mg of chloroquine diphosphate).

Studies on the concentration difference of CQ and CQM between plasma and serum were made on venous blood samples from 40 patients undergoing continuous CQ therapy for rheumatoid arthritis. They were between 41 and 65 years old and had been taking one oral dose of 0.16—0.25 g of chloroquine diphosphate daily for at least two months. The samples were withdrawn in the morning before the daily dose.

Studies on the effect of the time elapsed between the collection and centrifugation of blood samples were made on venous blood from six of these patients. Five samples each of heparin- and EDTA-stabilized whole blood were kept standing undisturbed at room temperature (23°C) for 0.5, 2, 4, 6 and 24 h before the plasma was separated by centrifugation. The plasma was then kept frozen (—65°C) until assayed.

The effect of variation of the centrifugal force was studied on the venous blood samples from three of the above six patients. Seven samples of heparin-stabilized whole blood from each patient were centrifuged 1—3 h after the collection at different centrifugal force for 10 min at room temperature (23°C) in a Laborfuge II centrifuge, (Heraeus Christ, Osterode, G.F.R.).

The sample tubes used for serum and EDTA-plasma came from Vacutainer (Becton-Dickinson, Rutherford, NJ, U.S.A.) and those used for heparin-plasma came from Venoject (Terumo, Tokyo, Japan).

Chemicals and reagents

Chloroquine, desethyl-chloroquine and the internal standard 6,8-dichloro-4-(1-methyl-4-diethylamino-butylamino)-quinoline, used in the liquid chromatographic determinations, were kindly donated by Sterling-Winthrop (Skärholmen, Sweden).

Lymphoprep^{R} was supplied from Nyegaard & Co. (Oslo, Norway) and Dextran 70, 6% in 154 mmol/l sodium chloride, came from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents were analytical grade from Merck (Darmstadt, G.F.R.). The aqueous solutions were prepared using high-purity water obtained from a Milli-Q deionized water system (Millipore, Bedford, MA, U.S.A.). Polypropylene tubes from Sarstedt (Malmö, Sweden) and siliconized glass tubes were used in the cell separations.

Liquid chromatography assay

The analyses of CQ and CQM in whole blood, serum, plasma and various cell suspensions were assayed using a high-performance liquid chromatographic (HPLC) method with fluorescence detection. Concentrations down to 0.5 nmol/l (0.15 ng/ml) can be determined with a relative standard deviation of 12%. A description of this method has previously been published by our laboratory [10].

This method requires an extraction step. Unfortunately, the recovery of CQ and CQM from aqueous solutions in the concentration range of 200—500 nmol/l was only about 70—80%. Therefore, for analysis of cell suspension an equal amount of EDTA-plasma (from a plasma pool free from CQ or CQM) was added before the extraction. The result was an almost 90% recovery of CQ and CQM from aqueous solutions of 200 nmol/l, as seen in Fig. 1.

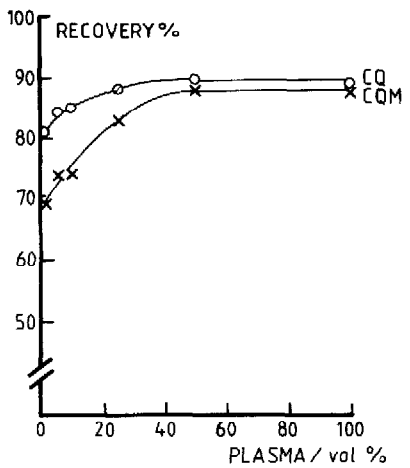

Fig. 1. Effect of different amounts of plasma in 154 mmol/l NaCl in phosphate buffer pH 7.0—7.4, on recovery of 200 nmol/l chloroquine (CQ) and desethyl-chloroquine (CQM) extracted as base with ethylene dichloride.

Isolation of blood cells

The isolation scheme is outlined in Fig. 2. All reagent solutions used contain 154 nmol/l NaCl in a phosphate buffer pH 7.0–7.4. This buffer was mixed with an equal amount of CQ- and CQM-free plasma (plasma-buffer) and was used as a suspension medium for cell counting and HPLC analysis.

On the basis of Bφyum's method for isolation of lymphocytes [11] and granulocytes, a technique was developed for the isolation of thrombocytes, granulocytes and erythrocytes. The aim was to obtain as pure cell fractions as possible with a reasonable recovery and not too many steps. The lymphocytes are in principle very interesting in experiments with blood cell uptake of drugs. However, the maximal recovery of lymphocytes was only about 20%. This was due to high losses of lymphocytes during the washing steps which were necessary to reduce the thrombocyte contamination.

Thrombocytes. A centrifugation isolation technique was employed. Eighteen millilitres of freshly drawn EDTA-blood were centrifuged at 100 *g*, for 15 min at 4°C. The plasma, containing the thrombocytes, was pipetted off into siliconized glass tubes using polypropylene pipettes. Since the plasma could not be completely removed without disturbing the buffy coat, about 10–15% of the plasma volume was left to avoid contamination with leucocytes. The

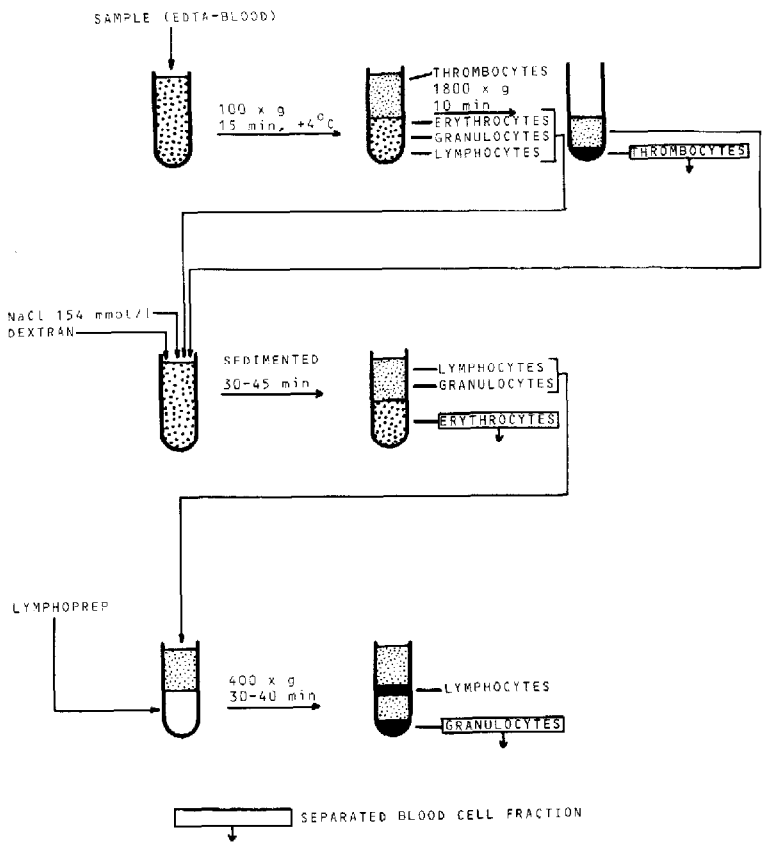

Fig. 2. Isolation of blood cells.

thrombocytes were centrifuged into a pellet at 1800 *g* for 10 min, leaving plasma which was free from thrombocytes above the pellet. This plasma was added to and mixed with the contents of the original blood tube for further separation of other blood cells. The pellets were resuspended in 5 ml of phosphate buffer containing 0.03 mol/l EDTA and 0.1 ml of plasma which was free from CQ and CQM. Contaminating erythrocytes and leucocytes were removed by centrifugation (100 *g*, 15 min) leaving a pure thrombocyte fraction. After centrifugation the thrombocytes were suspended in 6 ml of plasma-buffer.

Granulocytes. Whole blood, from which the main fraction of thrombocytes was removed by the above centrifugation technique, was prepared essentially as described by Bøyum [11]. Ten millilitres of blood were mixed with 4 ml of phosphate buffer, and then 2 ml of 6% Dextran 70. When the erythrocytes had sedimented (1 *g*, 30—45 min) the supernatant was pipetted off and carefully layered onto 8 ml of Lymphoprep. The tube was then centrifuged (400 *g*, 30—40 min). The supernatant was sucked up, leaving the granulocytes in the last 0.5—1.0 ml. To this fraction were admixed 5 ml of phosphate buffer containing 0.03 mol/l EDTA and 0.1 ml of plasma which was free from CQ and CQM. By centrifugation at 100 *g* for 10 min a granulocyte pellet was produced. Most of the contaminating lymphocytes and thrombocytes were left in the supernatant, which was removed. The washed granulocytes were suspended in 6 ml of plasma-buffer. The preparations contained 80—90% granulocytes, the contaminating cells being erythrocytes and lymphocytes.

Erythrocytes. The erythrocytes from the Dextran sedimentation step in the granulocyte separation above were centrifuged at 1000 *g* for 10 min and the supernatant and buffy coat were removed. The erythrocytes were washed three times with 7 ml of 154 mmol/l NaCl in phosphate buffer pH 7.0—7.4 and packed by centrifugation at 2000 *g*, 10 min. The erythrocyte fraction was almost free from other cells.

Cell counting

From the suspensions of the different blood cells, serial dilutions in three steps were made with the plasma-buffer. These diluted cell suspensions were counted in an automated blood-counting apparatus, Hemalog-8 (Technicon, Tarrytown, NY, U.S.A.), which is designed to count simultaneously erythrocytes, leucocytes and thrombocytes in blood samples. The purity of the blood cell fractions was also evaluated microscopically by manual counting in a cell chamber.

Analysis of CQ and CQM in cell fractions

The amounts of CQ and CQM in the different fractions were determined after adding 2 ml of 1 mol/l NaOH and freezing at −65° C to achieve cell lysis. The completeness of the cell lysis was controlled microscopically. The lysed cell suspensions were then extracted exactly as a plasma sample in the HPLC method [10]. Chromatograms from a granulocyte and a thrombocyte fraction are presented in Fig. 3 with a chromatogram of plasma standards for comparison. Fig. 3 also shows an unidentified metabolite present in the

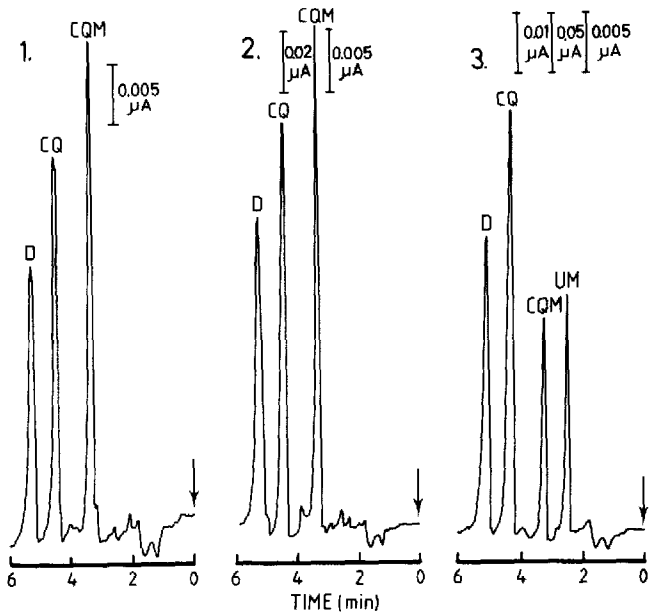

Fig. 3. Representative chromatograms from blood cell fractions and plasma standard of chloroquine (CQ) and desethyl-chloroquine (CQM). D = internal standard; UM = unidentified metabolite. (1) Plasma standard: 50 nmol/l CQ and CQM. (2) Granulocyte fraction: 130 nmol CQ per 10^9 cells and 27 nmol CQM per 10^9 cells from subject 1. (3) Thrombocyte fraction: 1.8 nmol CQ per 10^9 cells and 1.6 nmol CQM per 10^9 cells from subject 3.

thrombocyte fraction. The same metabolite is probably also present in plasma and urine [10].

In vitro uptake of CQ and CQM by blood cells

CQ and CQM, corresponding to about 1500—2000 nmol/l, were added to 25 ml of EDTA-stabilized whole blood from haematologically normal individuals. After incubation for 60 min at 37° C with mixing every 10 min, the blood cells were separated and the amount of CQ and CQM in the different blood cell fractions was determined as described above.

RESULTS

The concentration difference between plasma and serum

Fig. 4 shows the difference between serum and plasma concentrations of CQ and CQM in samples from 40 rheumatoid patients. The concentration of CQ in serum was two times higher, and the concentration of CQM in serum was four times higher than in plasma. The higher concentrations of CQ and CQM in serum might be explained as being due to the release of CQ and CQM from leucocytes and thrombocytes during the clotting process.

Sample handling

Fig. 5 shows that plasma should be separated from whole blood within 1—2 h to avoid enhanced plasma levels. In order to centrifuge the blood cells containing CQ and CQM, the centrifugal force must be above 1000 *g*, as seen in Fig. 6.

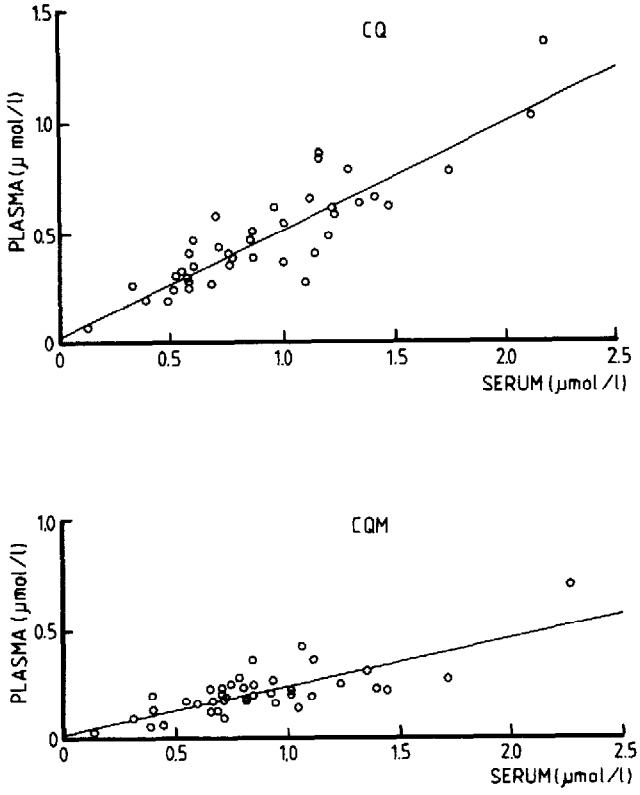

Fig. 4. Comparison between serum (X) and plasma (Y) from 40 patients on chloroquine therapy for chloroquine (CQ) and desethyl-chloroquine (CQM). Top graph: $Y = 0.50X + 0.024$, $r = 0.89$, $n = 40$. Bottom graph: $Y = 0.22X + 0.023$, $r = 0.76$, $n = 40$.

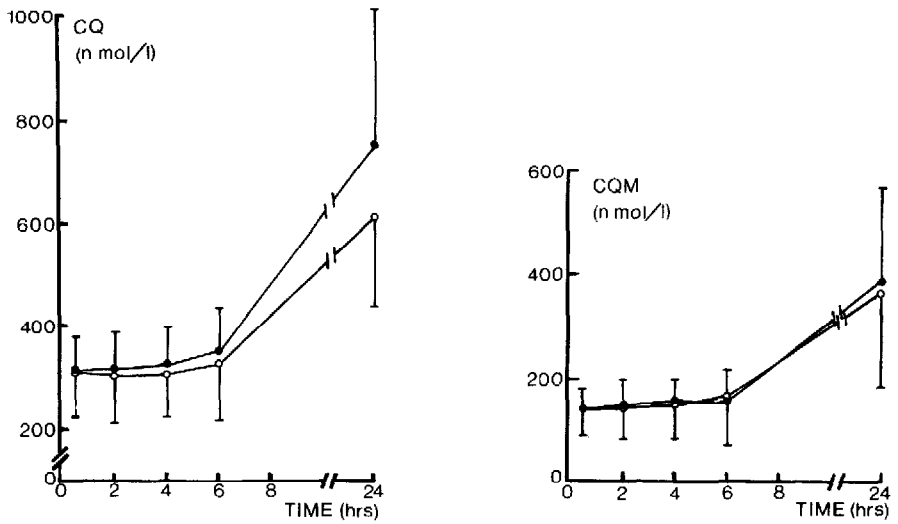

Fig. 5. The effect of storage of whole blood at room temperature (23°C) before centrifugation on the concentration of CQ (upper) and CQM (lower) in plasma. The length of the bar corresponds to the standard deviation. (•) Heparin-plasma; (○) EDTA-plasma.

TABLE I

IN VIVO BLOOD CELL, PLASMA, AND WHOLE BLOOD CONCENTRATIONS OF CHLOROQUINE (CQ) AND DESETHYL-CHLOROQUINE (CQM) (A) FROM THREE HEALTHY SUBJECTS GIVEN AN ORAL DOSE OF 1000 mg OF CHLOROQUINE PHOSPHATE AND (B) AFTER INCUBATION OF WHOLE BLOOD IN VITRO WITH 1500—2000 nmol/l CQ AND CQM FOR 60 min

Samples were taken 7 h after ingestion for the in vivo measurements. Subjects 1 and 2 are the same in vivo and in vitro.

	Subjects														
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	No. of cells in whole blood ($\times 10^9$ cells/l)			CQ						CQM					
				nmol/10^9 cells			nmol/l whole blood*			nmol/10^9 cells			nmol/l whole blood*		
(A) In vivo															
Blood cells															
Granulocytes	4.5	3.3	3.6	130	221	105	585	729	378	27	24	17	122	79	61
(Lymphocytes	2.7	1.8	2.7)	—	—	—	—	—	—	—	—	—	—	—	—
Thrombocytes	259	254	205	3.9	2.4	1.8	1010	610	387	2.6	1.7	1.6	673	432	328
Erythrocytes	4100	5000	5100	0.101	0.040	0.030	414	200	153	0.046	0.016	0.014	189	80	71
Plasma**,***				—	—	—	547	246	149	—	—	—	224	57	62
Whole blood***				—	—	—	2880	2050	1350	—	—	—	1100	707	507
Recovery+				—	—	—	2560 (89%)	1790 (87%)	1070 (79%)	—	—	—	1210 (110%)	650 (92%)	520 (103%)
(B) In vitro															
Blood cells															
Granulocytes	2.9	3.2	5.9	233	120	71	676	384	419	75	29	27	218	93	159
(Lymphocytes	4.7	2.0	5.1)	—	—	—	—	—	—	—	—	—	—	—	—
Thrombocytes	251	250	385	1.7	1.5	1.2	440	375	462	4.7	3.0	2.7	1180	750	1040
Erythrocytes	4300	5400	4900	0.053	0.044	0.030	207	238	162	0.065	0.046	0.044	280	225	216
Plasma**,***				—	—	—	268	241	95	—	—	—	318	233	173
Whole blood***				—	—	—	1870	1440	1460	—	—	—	2080	1570	1770
Recovery§				—	—	—	1590 (85%)	1240 (86%)	1140 (78%)	—	—	—	2000 (96%)	1300 (83%)	1590 (90%)

*The numbers in these columns represent the contribution of the fractions to the total whole blood concentration.
**The concentration of CQ and CQM in plasma is corrected for the hematocrit.
***Plasma and whole blood concentration of CQ and CQM were analysed by the method described in ref. 10.
§The recovery in whole blood is calculated by adding the concentration contribution of the different blood cells to that of plasma. This value is compared with the total concentration in whole blood.

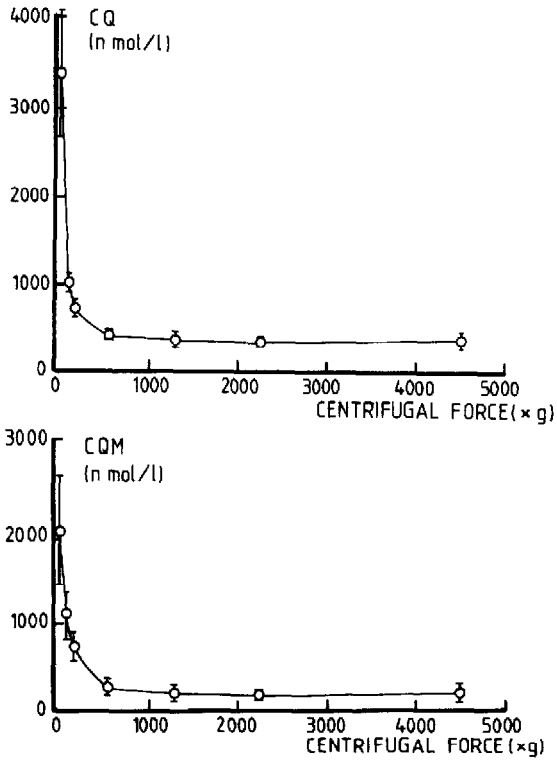

Fig. 6. The effect of variation of the centrifugal force (*g*) on CQ (upper) and CQM (lower) concentration in plasma. Centrifugation time 10 min. The length of the bar corresponds to the standard deviation.

Recovery of the cell fractions

The recovery of the different cell types from whole blood with the presented separation technique was for thrombocytes 64 ± 14%, for granulocytes 47 ± 8%, and for erythrocytes 73 ± 5%, calculated from seven experiments.

In vivo uptake of CQ and CQM by blood cells

Four different dilutions were made from the blood cell fractions of erythrocytes, granulocytes and thrombocytes. Cell counts and CQ and CQM analyses were made on these dilutions and plotted as shown, for granulocytes and thrombocytes from one subject, in Fig. 7. This demonstrates the good positive linear correlation between the cell counts for thrombocytes and granulocytes and the HPLC determinations in blood cells, and confirms that the measuring ranges are suitable for determination of CQ and CQM in blood cells.

The slope of the regression lines provides the CQ and CQM amount per 10^9 cells. This, together with the count of erythrocytes and thrombocytes in the original whole blood sample, gives the contribution to the total blood CQ and CQM concentrations from these cells. In a similar manner, the granulocyte contribution is calculated from the total leucocyte count and from the differential counting.

Table I shows the amounts and concentrations of CQ and CQM in different blood cells from the three subjects who had taken one oral CQ dose. The large

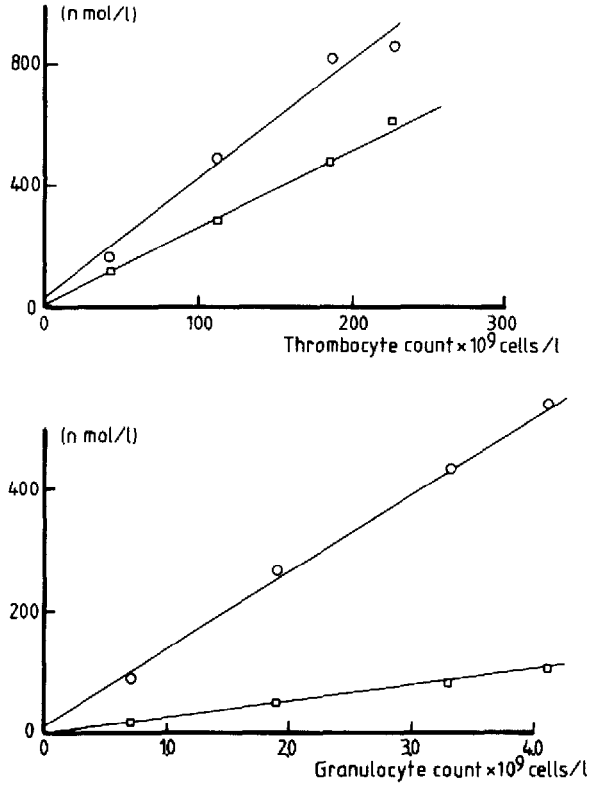

Fig. 7. Relationship between thrombocyte and granulocyte cell counts and chloroquine (CQ) and desethyl-chloroquine (CQM) concentration. Top panel: for CQ (○), $Y = 3.92 \cdot 10^{-9} X + 35$, $r = 0.986$; for CQM (□), $Y = 2.60 \cdot 10^{-9} X + 6$, $r = 0.998$. Bottom panel: for CQ (○), $Y = 130 \cdot 10^{-9} X + 9$, $r = 0.999$; for CQM (□), $Y = 27 \cdot 10^{-9} X - 1.8$, $r = 0.999$.

amount of CQ and CQM in granulocytes and thrombocytes is especially noteworthy.

Of the total concentration in whole blood, 84 ± 5% of CQ and 96 ± 9% of CQM were recovered in the different fractions in vivo as well as in vitro (Table I). However, there are many steps in the cell separation procedure. The error of cell counts and differential counts is 5—10% and the error in the chromatographic analysis is about 5%. The systematic recovery deficit could be explained by the assumption that the lymphocytes contain CQ and CQM.

In vitro uptake of CQ and CQM by blood cells

The uptake in vitro of CQ and CQM in blood cells was about the same as in vivo. The results are also presented in Table I.

DISCUSSION

In this study we have found large amounts of CQ and CQM in thrombocytes and granulocytes in vivo as well as in vitro. In other investigations [12] it has been shown that erythrocytes from monkeys infected with *Plasmodium*

falciparum bind CQ to a high degree. In uninfected erythrocytes such high binding could not be seen. It is a common practice that the erythrocyte cell concentration of drugs is calculated from analysis on serum and whole blood [13]. If the blood/plasma distribution ratio exceeds 1.0, the drug is considered to be distributed in the erythrocytes [14]. As we have shown, other blood cells should also be taken into consideration.

A very important question in clinical practice is which biological material is the most suitable for the analysis of drugs. Concerning the determination of CQ and its metabolite CQM we may conclude that heparin- or EDTA-plasma are the most suitable biological materials, if they are depleted of thrombocytes and granulocytes within 1—2 h after the sampling by centrifugation at high speed (>1000 *g*, 15 min).

As reported in a previous paper [15], we found no change in CQ and CQM values when the plasma samples were stored for up to eleven months at −20° C. Repeated freezing and thawing, five times, of a plasma pool sample from patients undergoing treatment with CQ did not result in any change of CQ and CQM concentration.

In a recent investigation [16] it was found that the concentration of the antiarrhythmic drug quinidine was higher in serum than in plasma. Quinidine and CQ are both 4-aminoquinolines and therefore could have similar binding properties to the thrombocytes and granulocytes. The quinidine results could be explained in the same way as the CQ and CQM serum/plasma discrepancy.

The uptake of CQ and CQM by blood cells, demonstrated in this investigation, could be important for the effect of CQ on the inflamed tissue in rheumatoid arthritis. The effect of CQ is not clearly understood, but an accumulation of CQ has been shown in the lysosomes of many different cells [17], including fibroblasts [18, 19], from many different tissues. Both granulocytes and thrombocytes possess a large amount of lysosomes [20, 21]. It has also been shown that CQ has a stabilizing effect on the lysosome membrane [22], inhibits the transformation of lymphocytes [23], and reduces the activity of several lysosomal enzymes [18, 24]. The transportation of CQ with the granulocytes to the inflamed tissue would improve the possibility for a higher concentration locally, thereby making an enhanced action of CQ possible.

In conclusion, the study has shown the importance of sample handling and choice of material for analysis of drugs in blood. The technique used for estimation of the amount of CQ and CQM in blood cells could easily be adapted for other drugs provided suitable analytical methods are available.

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