Journal of Chromatography, 274 (1983) 111—120
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1585

DETERMINATION OF CHLOROQUINE AND ITS MAJOR METABOLITE IN BLOOD USING PERFLUOROACYLATION FOLLOWED BY FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

FREDERICK C. CHURCHILL, II*, DWIGHT L. MOUNT and IRA K. SCHWARTZ

Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 30333 (U.S.A.)

(First received August 11th, 1982; revised manuscript received October 27th, 1982)

SUMMARY

Tandem fused-silica capillary gas chromatographic methods for the determination of chloroquine and its major metabolite, desethylchloroquine, are described. Method A employs a single extraction step and internal standardization to permit rapid, precise analyses for chloroquine in whole blood. Method B, employing derivatization with pentafluoropropionic anhydride, can then be applied to the extract to allow qualitative and quantitative confirmation of chloroquine and sensitive, precise quantification for desethylchloroquine. The detection limit for chloroquine in blood is 5 ng/ml by both methods; the limit for desethylchloroquine is 15 ng/ml. Excellent precision is achieved by the methodology, partly due to the use of separate internal standards for the two analytes, each internal standard being a close analogue of the corresponding analyte. Data are presented which demonstrate the increase over time of metabolite relative to unchanged chloroquine found in the blood of a volunteer undergoing a chemoprophylactic regimen of chloroquine.

INTRODUCTION

A variety of analytical approaches have been employed to quantify chloroquine in body fluids [1—12] in support of investigations into its use as an antimalarial and antirheumatic drug. Four of these methods permit quantitation of both chloroquine and its major metabolite, desethylchloroquine, in blood, three using high-performance liquid chromatography (HPLC) [6,10,11] and the other gas—liquid chromatography [9]. In the present work we report a procedural sequence which permits the analyst to rapidly, sensitively, and precisely determine chloroquine concentration in blood and then, if desired,

0378-4347/83/$03.00

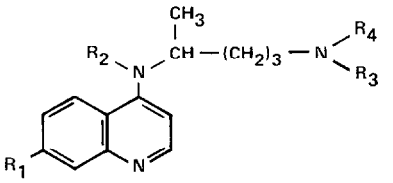
employ derivatization with pentafluoropropionic anhydride to confirm chloroquine identity and quantity while quantifying desethylchloroquine.

EXPERIMENTAL

Standards

Chloroquine diphosphate was purchased from Sigma (St. Louis, MO, U.S.A.)*. This material was dried under vacuum over phosphorus pentoxide and the melting point determined using a Fisher—Johns apparatus calibrated with Fisher TherMetric standards before its use as a standard. The melting point range was 192.1—193.3°C. Desethylchloroquine base and iodoquine diphosphate were supplied by the Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). The isopropyl analogue of desethylchloroquine was provided by Mr. William Ellis of the Walter Reed Army Institute of Research (Washington, DC, U.S.A.).

The internal standard mixture referred to in the analysis (see below) contains roughly 6.6 μg/ml iodoquine (as base) and 5.1 μg/ml isopropyl analogue of desethylchloroquine (Fig. 1, compounds IIa and IVa, respectively) in 0.002 *M* aqueous hydrochloric acid.



Designation	R_1	R_2	R_3	R_4	t_R(min)
Ia	Cl	H	CH_2CH_3	CH_2CH_3	8.6
Ib	Cl	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	CH_2CH_3	CH_2CH_3	7.0
IIa	I	H	CH_2CH_3	CH_2CH_3	11.6
IIb	I	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	CH_2CH_3	CH_2CH_3	9.0
IIIa	Cl	H	H	CH_2CH_3	8.0
IIIb	Cl	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	CH_2CH_3	7.8
IVa	Cl	H	H	$CH(CH_3)_2$	8.3
IVb	Cl	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	$\overset{O}{\overset{\|}{C}}CF_2CF_3$	$CH(CH_3)_2$	8.1

Fig. 1. Structures of chloroquine, desethylchloroquine, internal standard compounds, and their pentafluoropropionyl derivatives together with retention times (t_R) under the chromatographic conditions employed in this study. The final column temperature is 230°C for compounds Ia—IVa, 220°C for compounds Ib—IVb.

*Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

Pentafluoropropionylchloroquine was synthesized by adding 100 μl of pentafluoropropionic anhydride and 25 μl of triethylamine to a 10.3-mg quantity of chloroquine base in a 15-ml screw-cap centrifuge tube. The tube was capped and sealed using PTFE tape and the mixture heated to 90°C for 30 min. The mixture was cooled, diluted with 3 ml of benzene, and partitioned with 0.5 ml of 5% ammonia. The benzene layer was transferred to a separate centrifuge tube and this stock diluted as necessary for qualitative chromatographic studies. Gas chromatographic—mass spectrometric (GC—MS) analysis showed the presence of a small amount of unreacted chloroquine and an earlier eluting peak due to a large quantity of material containing an apparent molecular ion at *m/e* 465 together with major fragments at *m/e* 450 (loss of CH_3), *m/e* 318 (loss of $CF_3CF_2CO^{\bullet}$) and *m/e* 86 [$CH_2{=}\overset{+}{N}(CH_2CH_3)_2$]. This pattern is definitive for the proposed compound.

Bis-pentafluoropropionyldesethylchloroquine was similarly synthesized. The benzene extract contained primarily the compound sought together with a small quantity of the expected monoacylated product. GC—MS analysis showed no peaks for the molecular ion expected, but prominent peaks at *m/e* 436 (loss of $CF_3CF_2CO^{\bullet}$), *m/e* 351 [loss of $^{\bullet}CH_2CH_2CH_2N(CH_2CH_3)(COCF_2CF_3)$], *m/e* 260 [$CH_3\overset{+}{C}HCH_2CH_2CH_2N(CH_2CH_3)(COCF_2CF_3)$], and *m/e* 204 [$CH_2{=}\overset{+}{N}(CH_2CH_3)(COCF_2CF_3)$]. These fragments establish the structure of the compound proposed.

Reagents

Hexane was glass-distilled, available from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The octane and *n*-decylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.). Human blood used for standards was freshly collected using acid—citrate—dextrose (ACD) as an anticoagulant. Pentafluoropropionic anhydride was supplied by Pierce (Rockford, IL, U.S.A.). Pyridine was from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and was distilled over sodium hydroxide. All other chemicals used were of reagent grade or better.

Equipment

Gas chromatography—mass spectrometry. Pentafluoropropionyl derivatives of chloroquine and desethylchloroquine were characterized with a Hewlett-Packard (Avondale, PA, U.S.A.) Model 5992 gas chromatograph—mass spectrometer—computer system. Studies were performed using a 1.83 m × 2.0 mm I.D. glass column packed with 5% OV-101 on 100—120 mesh Gas Chrom Q and a helium flow-rate of 16 ml/min; injector, column, jet separator, and source temperatures were 250, 250, 250, and 150°C, respectively, and electron-impact ionization was performed at 70 eV.

Gas chromatography. A Hewlett-Packard Model 5880A gas chromatograph equipped with a capillary injector and a nitrogen-specific detector was used in this study. A 25 m × 0.3 mm I.D. fused-silica capillary column was used which had been siloxane deactivated and coated with OV-1 by the manufacturer (Part No. 19091-62325; Hewlett-Packard). On-column injection was used. The front 1.5-m portion of the column was stripped of liquid phase using methylene chloride. When the injection of a large number of samples had degraded column performance, removal of a 25-cm portion of the front of the

column restored efficiency. After four to five such "surgeries," the front end of the column was again stripped of liquid phase. The injector heater was not turned on, so that the injector temperature remained below the column initial temperature of 125°C. Detector temperature was 290°C. The column temperature remained at the 125°C initial temperature for 1 min and then was programmed to increase at 20°C/min to 230°C for Method A and 220°C for Method B. Helium carrier gas flow-rate was in the range of 3—4 ml/min. The syringe used for on-column injection was Part No. 9301-0562 (Hewlett-Packard), which is fitted with a needle 125 mm in length.

Method A

Conical centrifuge tubes (15 ml) were fortified with 100-μl volumes of 0.001 *N* aqueous hydrochloric acid solutions containing the following quantities of chloroquine and desethylchloroquine expressed in ng as base: 0.0, 0.0; 99.1, 119.7; 198.2, 239.4; 247.7, 359.2; 495.4, 478.9; and 743.1, 598.6. To each tube were added 100 μl of the internal standard mixture (see standards), 1 ml of ACD blood, and 2 ml of deionized water. A 1-ml quantity of each (heparinized) blood sample was pipetted into a 15-ml centrifuge tube to which a 100-μl quantity of internal standard mixture had been added. A 2-ml rinse of deionized water served to complete the transfer of blood sample into the centrifuge tube. Sample and standard alike were mixed using a 5-sec vortex. To each tube were added 3 ml of hexane and 0.5 ml of 5 *N* sodium hydroxide solution. Each tube was vortexed for 30 sec, followed by 20 min shaking on a reciprocal shaker. Two drops of isoamyl alcohol were added, followed by 5 min centrifugation, to aid in the separation of layers. The bulk of each hexane layer was transferred to a silanized [5] 15-ml centrifuge tube fitted with a cap containing a PTFE insert. The hexane was evaporated using a 60°C water bath and a stream of dry nitrogen. Each sample and standard were reconstituted using 25 μl of *n*-octane. On-column injections were made after drawing up 1.2 μl of 200 ng/ml *n*-decylamine in octane, about 1.8 μl of air, and 1.4 μl of sample or standard. After injection an 0.8-μl quantity of liquid remained in the syringe.

Method B

To the remainder of each of the concentrated extracts left after the injection of Method A, about 2 ml of benzene were added and then evaporated using a stream of dry nitrogen. This benzene addition—evaporation step was repeated to ensure dryness of the residue. To each tube were added 50 μl of pentafluoropropionic anhydride and 10 μl of dry pyridine. The tubes were sealed using PTFE tape and heated to 60°C for 30 min. Each tube was then cooled and 2 ml of hexane added, followed by 5-sec vortexing. A 2-ml quantity of 1 *N* ammonia was added and each tube vortexed for 30 sec. The bulk of the hexane layer was transferred by a Pasteur pipet to a 3-ml Reacti-vial. The hexane was evaporated using an aluminum block at 60°C with hood air-flow passing over the vials. Each sample and standard was then reconstituted in 25 μl of *n*-octane with vortexing, and a 1—2 μl quantity was injected onto the capillary column.

RESULTS AND DISCUSSION

Characterization of Methods A and B

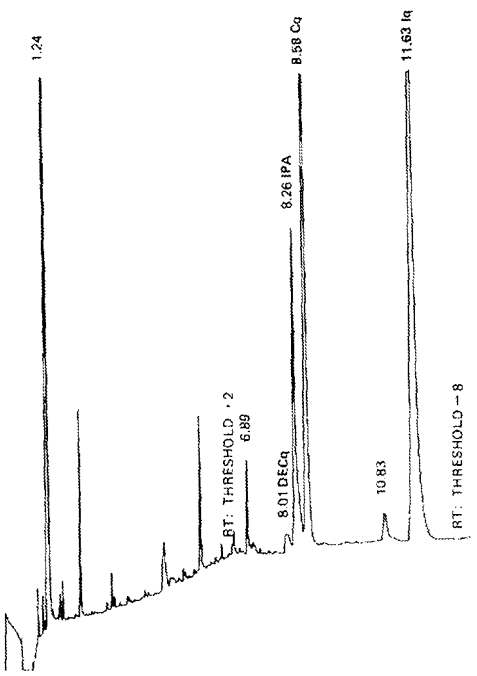
The methodology presented herein is designed to provide a rapid, accurate value for chloroquine concentration in whole blood and blood fractions while presenting the option for confirmation of the chloroquine value and quantification of desethylchloroquine, all using a single 1-ml sample. The first phase of the analysis, referred to as Method A, is a modification of our previously presented procedure [12]. Iodoquine has been substituted for bromoquine as internal standard, since pentafluoropropionylbromoquine (PFP-bromoquine) and bis-PFP-desethylchloroquine (Fig. 1, compound IIIb) would coelute in the second phase of the determination. Also, *n*-decylamine is not added directly to the *n*-octane solvent for the concentrated extract but is instead used in an *n*-octane solution to provide a "solvent flush" [13], as described in the Experimental section, to minimize adsorption both within the syringe and on the front of the capillary column. Failure to use the solvent-flush technique appreciably lowered sensitivity of the method.

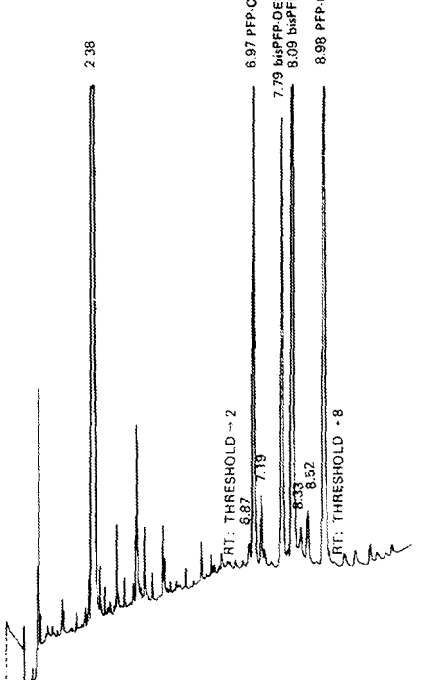
Method B consists of a derivatization and extraction step performed subsequent to Method A. The perfluoropropionated compounds exhibit no evidence of adsorption under the GC conditions employed in the present study. Comparison of the results from the two methods can be made by perusal of Figs. 2 and 3 and Tables I and II.

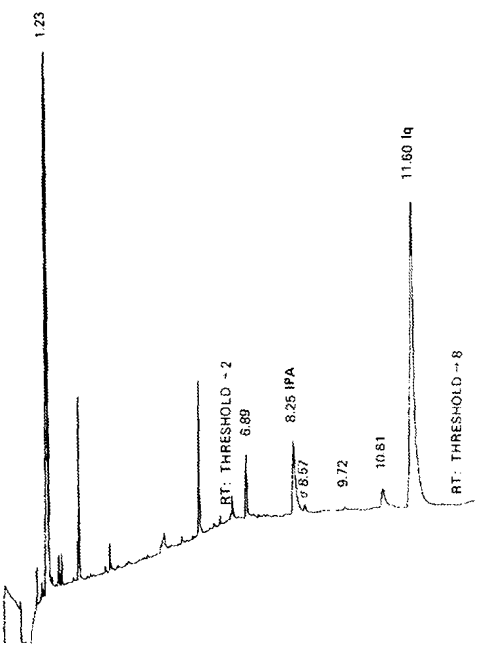
The column performance was less than optimal when the chromatograms in Fig. 2 were run. Tailing is evident not only for compound IVa but also for iodoquine (IIa). Chloroquine peak shape is good, with chloroquine eluting on the tail of IVa. Removal of 25 cm of the solvent-stripped front end of the column followed by reinstallation yielded the much-improved column performance seen in Fig. 3. The discrimination of the column against desethylchloroquine relative to chloroquine is evident in Figs. 2 and 3. On the other hand, as might be expected, the peak shapes of the PFP derivatives, seen in the chromatographic traces on the right half of Fig. 2, are not adversely affected by the factors which cause tailing in the underivatized compounds. The internal standard peak heights for the chromatogram of the blank standard in Fig. 2 are less than for the standard containing both chloroquine and desethylchloroquine. Some losses of amine analytes and standards occur, presumably by adsorption, during the analysis, and such losses have the greatest impact on internal standard peak heights when no analyte compounds are present to share adsorptive losses.

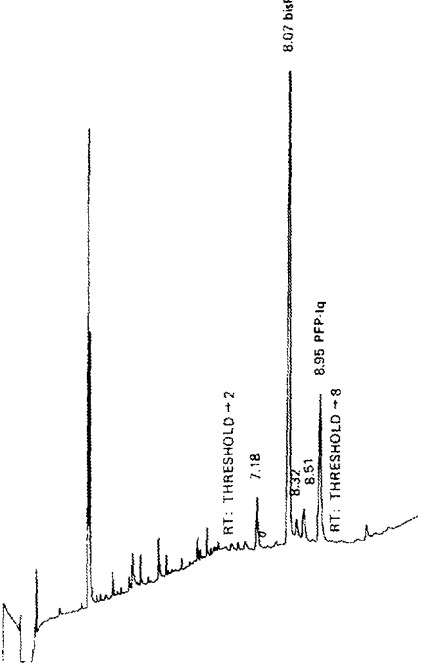
The small chloroquine peak seen for the blank by Method A is due to non-negligible but reproducible "ghosting," the magnitude of which is only slightly affected by the quantity of chloroquine in the immediately preceding injection [12].

Results in Table I show that Method B exhibits marginally better linearity and precision than Method A. The slopes of the two regression lines are nearly identical. The relative standard deviation (R.S.D.) at the 247.7 ng/ml level for chloroquine is better than 2% for both methods. The detection limit for both methods is conservatively calculated to be 5 ng/ml of chloroquine in blood. It


1.24
RT: THRESHOLD • 2
6.89
8.01 DECq
8.26 IPA
8.58 Cq
10.83
11.63 Iq
RT: THRESHOLD → 8


2.38
RT: THRESHOLD → 2
6.87
7.19
6.97 PFP-Cq
7.79 bisPFP-DECq
8.09 bisPFP-IPA
8.33
8.52
8.98 PFP-Iq
RT: THRESHOLD • 8


1.23
RT: THRESHOLD • 2
6.89
8.25 IPA
8.67
9.72
10.81
11.60 Iq
RT: THRESHOLD → 8


RT: THRESHOLD → 2
7.18
8.07 bisPFP-IPA
8.37
8.51
8.95 PFP-Iq
RT: THRESHOLD → 8

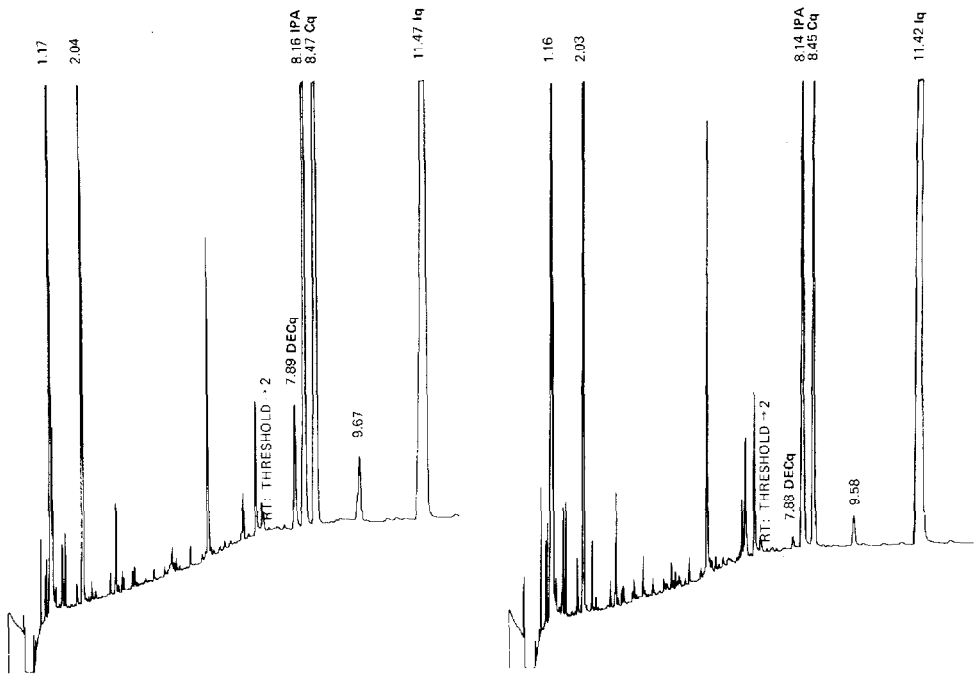


Fig. 3. Chromatograms resulting from the application of Method A to a blood standard and a serum sample. Note the efficiency and lack of activity of the column exhibited by these chromatograms. The left trace corresponds to a blood standard containing 247.7 ng of chloroquine, 359.2 ng of desethylchloroquine, and internal standard compounds. The right trace corresponds to a serum sample found to contain 77.2 ng/ml of chloroquine. Because of a marginally higher flow-rate, retention times are slightly shorter than those in Figs. 1 and 2. Abbreviations are as in Fig. 2.

should be emphasized that the same standards were carried first through Method A and then through Method B so that all corresponding values are directly comparable. Compound IVa is central to the tandem methodology in that it serves as a carrier desorber and improves chloroquine chromatography in Method A and then serves as an analogue internal standard for desethylchloroquine in Method B.

Data in Table II illustrate excellent linearity for the desethylchloroquine determination. Four replicate injections at the 359.2 ng/ml standard level show that repeatability of the chromatography is excellent. The relative standard deviation found for four standards at the 359.2 ng/ml level was 4.17%. It is to be expected that the precision would be less for this more basic, more readily adsorbed analyte than for chloroquine. The detection limit is 15 ng/ml of desethylchloroquine in blood.

Fig. 2. Chromatograms resulting from the application of Method A and Method B to standards. Upper chromatograms correspond to a standard containing 247.7 ng/ml of chloroquine (Cq) and 359.2 ng/ml of desethylchloroquine (DECq) in blood together with added internal standards. Lower chromatograms correspond to a blank sample containing added internal standards only. Left chromatograms result from Method A, right chromatograms from Method B. Peaks: IPA = isopropyl analogue of desethylchloroquine; Iq = iodoquine; and PFP = the pentafluoropropionyl derivative of a given compound.

TABLE I

STANDARD CURVE DATA FOR DETERMINING CHLOROQUINE IN BLOOD BY METHOD A AND METHOD B

Cq added x (ng)	Method A		Method B	
	Cq/internal standard peak area ratio	Cq calculated* x' (ng)	Cq/internal standard peak area ratio	Cq calculated*** x' (ng)
0.0	0.0103	7.00	0.0000	−2.20
99.1	0.2215	99.4	0.2253	98.6
198.2	0.4278	189.6	0.4587	203.1
247.7	0.5422	239.6**	0.5615	249.1§
	0.5634	248.9	0.5536	245.6
	0.5425	239.8	0.5468	242.6
	0.5445	240.6	0.5522	245.0
495.4	1.1456	503.5	1.1144	496.6
743.1	1.6904	741.7	1.6620	741.7

*Calculated from the least-squares line, $y=mx + b$ (m = 0.002287; b = −0.005710); r_6^2 = 0.99942.
**R.S.D. (interstandard, n = 4) =1.84%.
***Calculated from the least-squares line, $y=mx + b$ (m = 0.002234; b = 0.004914); r_6^2 = 0.99990.
§ R.S.D. (interstandard, n = 4) = 1.09%; R.S.D. (intrastandard, 4 injections) = 0.94%.

TABLE II

STANDARD CURVE DATA FOR DETERMINING DESETHYLCHLOROQUINE (DECq) IN BLOOD BY METHOD B

DECq added x (ng)	DECq/internal standard peak area ratio	DECq calculated* x' (ng)
0.0	0.0000	1.37
119.7	0.1275	125.0
239.4	0.2380	232.2
359.2	0.3681	358.4**
	0.3634	353.9
	0.3848	374.6
	0.3476	338.5
478.9	0.4911	477.8
598.6	0.6202	603.0

*Calculated from the least-squares line, $y=mx + b$ (m = 0.001031; b = −0.001409); r_6^2 = 0.99956.
**R.S.D. (interstandard, n=4) = 4.17%; R.S.D. (intrastandard, 4 injections) = 0.63%.

Should studies indicate that chloroquine is, in general, substantially more effective than its metabolite against malaria parasites, the very precise blood-level value for chloroquine rapidly available using Method A might well provide sufficient information for most purposes. In cases where confirmation of chloroquine presence and quantity and/or measurement of desethylchloroquine levels is of interest, Method B provides values with excellent precision.

The precision of the analytical results within the concentration range of application of Methods A and B is better than most, if not all, chloroquine assays published to date. This combination of methods employs a separate close-analogue internal standard for each of the two analytes so that compensation during prechromatographic steps is highly effective. The precision for replicate injections of a given standard demonstrates that the structural similarities between internal standards and analytes together with the inert chromatographic system conditions provide a high order of internal compensation during chromatography.

Application of the methodology to clinical studies

The demonstration of an appreciable chloroquine level in the serum of a patient who contracted falciparum malaria while under chloroquine chemoprophylaxis (Fig. 3) showed, in concert with other evidence, that the parasites causing the disease were indeed chloroquine resistant. In a volunteer study, the percentage of desethylchloroquine relative to total determined, chloroquine-derived species in the subject increased substantially over the first eight weeks of a chemoprophylactic regimen. In general, however, the quantity of chloroquine in the blood at each of the observed time intervals subsequent to weekly tablet ingestion increased over the eight-week period. The data are summarized in Table III.

In summary, the methodology described above provides a valuable set of tools for continuing studies of chloroquine chemoprophylaxis and chemotherapy, particularly as they apply to chloroquine-resistant strains of *Plasmodium falciparum*.

TABLE III

WHOLE BLOOD CONCENTRATIONS OF CHLOROQUINE (Cq) AND DESETHYLCHLOROQUINE (DECq) AT INTERVALS DURING THE FIRST EIGHT WEEKS OF A CHLOROQUINE DIPHOSPHATE CHEMOPROPHYLACTIC REGIMEN (5 mg/kg AS BASE/WEEK)

Week	Time (h)	Cq (ng/ml)	DECq (ng/ml)	$\frac{\text{DECq (100)}}{\text{Cq + DECq}}$ (%)
1	8	343	57.6	14.4
	24	243	54.0	18.2
	72	131	15.5	10.6
	168	61	6.8	10.0
3	8	586	286	32.8
	24	352	198	36.0
	72	213	110	34.1
	168	122	90.5	42.6
8	8	631	377	37.4
	24	349	269	43.5
	72	226	193	46.1
	168	164	119	42.0

ACKNOWLEDGEMENT

These studies were supported in part by the Scientific Working Group on Chemotherapy of Malaria, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1 J.L. Holtzman, Anal. Biochem., 13 (1965) 66.
2 L. Kazyak, in I. Sunshine (Editor), Manual of Analytical Toxicology, CRC Press, Cleveland, OH, 1971, pp. 86—88.
3 C.W. Vogel and E. Konigk, Tropenmed. Parasit., 26 (1975) 278.
4 A. Viala, J.P. Cano and A. Durand, J. Chromatogr., 111 (1975) 299.
5 M.A. Staiger, P. Nguyen-Dinh and F.C. Churchill, II, J. Chromatogr., 225 (1981) 139.
6 Y. Bergqvist and M. Frisk-Holmberg, J. Chromatogr., 221 (1980) 119.
7 M. Bonini, F. Mokofio and S. Barazi, J. Chromatogr., 224 (1981) 332.
8 A. Viala, E. Deturmeny, M. Estadieu, A. Durand and J.P. Cano, J. Chromatogr., 224 (1981) 503.
9 Y. Bergqvist and S. Eckerbom, J. Chromatogr., 226 (1981) 91.
10 G. Alván, L. Ekman and B. Lindström, J. Chromatogr., 229 (1982) 241.
11 N.D. Brown, B.T. Poon and J.D. Chulay, J. Chromatogr., 229 (1982) 248.
12 F.C. Churchill, II and M.A. Staiger, Analytical Division, 183rd National Meeting of the American Chemical Society, Las Vegas, Nevada, March 31, 1982, Abstract No. 92.
13 H. Brötell, J. Chromatogr., 196 (1980) 489.