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Determination of quinine in serum, plasma, red blood cells and whole blood in healthy and *Plasmodium falciparum* malaria cases by high-performance liquid chromatography

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ABSTRACT

A normal-phase high-performance liquid chromatographic method using dichloromethane–methanol–1 *M* perchloric acid (100:9:0.4, v/v) at a flow-rate of 0.8 ml/min on a Zorbax-Sil column with fluorescence detection has been developed for the separation of quinine and quinidine from other antimalarials. Within-day and day-to-day coefficients of variation averaged 0.74 and 7.56%, respectively. The extraction recovery of quinine for plasma, serum, red blood cells and whole blood (filter paper) was 88.13, 87.12, 78.0 and 77.5%, respectively. The method is capable of separating quinine from dihydroquinine, a compound usually found as an impurity in authentic quinine samples. The method has been used for the determination of quinine in plasma, serum, red blood cells and whole blood (filter paper) of six healthy and twenty *Plasmodium falciparum* malaria cases. The average quinine concentration in *P. falciparum* malaria cases was three to four times higher than that in healthy volunteers. Quinine was absorbed much less in red blood cells than in plasma or serum.

INTRODUCTION

The widespread emergence of chloroquine-resistant falciparum malaria has renewed interest in the use of quinine for its treatment [1]. Quinine is still the drug of choice for severe and complicated *Plasmodium falciparum* malaria [2], but it has a variety of side-effects including cinchonism, cardiovascular toxicity, gastrointestinal irritation and hypersensitivity reactions [3]. Chongsuphajaisiddhi *et al.* [4] indicated a significant contraction of the volume of distribution of quinine in children who had *P. falciparum* malaria compared with uninfected children. White *et al.* [5] provided evidence of detectable levels of quinine in plasma after day 3, *i.e.* at the time when the fever usually disappears. Determination of the drug concentration in different body fluids is important for proper treatment of malaria cases, as well as in the investigation of dose-dependent adverse reactions [6]. Quinine has usually been measured in biological fluids by extraction fluorescence methods that lack selectivity and sensitivity [7,8]. Recently, high-performance liquid chromatography (HPLC) has been used for the determination of quinine in serum [9], plasma [10,11] and

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whole blood [12,13]. Edstein *et al.* [9] showed that quinine levels are different in capillary and venous blood, and Hellgren *et al.* [12] reported them to be the same.

This paper describes a simple and sensitive HPLC method for the detemrination of quinine in plasma, serum, red blood cells and whole blood (filter paper method) of healthy and *P. falciparum* malaria cases, and resolves the controversy concerning quinine levels in capillary and venous blood samples.

EXPERIMENTAL

Chemicals and standards

HPLC-grade dichloromethane, methanol and dichloroethane were obtained from Sisco Research Labs. (Bombay, India). All other reagents were of analytical grade and were used without further purification.

Quinine and quinidine were supplied by Sigma (St. Louis MO, USA). A stock standard solution of quinine sulphate was prepared in methanol (100 μg of quinine base per ml of methanol). Intermediate working standard solutions covering the concentration range 0.25–20 μg/ml) reported by Edstein *et al.* [9] were prepared by diluting the stock standard solution with methanol. Solutions were stored at 4°C.

Instrumentation and chromatographic conditions

A Waters 840 HPLC system (Waters Assoc., Milford, MA, USA) consisting of a 510 pump, a 490 programmable multiwavelength UV detector operated at 254 nm and a Shimadzu fluorescence RF-530 detector operated at an excitation wavelength of 350 nm with a 418-nm emission cut-off filter, a WISP 712 automatic sample injector and a digital 380 data station with digital printer was used for analysis. The detectors were operated in series. The mobile phase was dichloromethane–methanol–1 *M* perchloric acid (100:9:0.4, v/v) and was pumped at a flow-rate of 0.8 ml/min through a Zorbax-Sil column (250 mm × 4.6 mm I.D., 5 μm particle size; DuPont, Wilmington, DE, USA) for the successful separation of all antimalarials under study. The mobile phase was filtered and degassed by ultrasonication (Decon FS 100, Hove, UK) before use. Chromatography was performed at ambient temperature.

Subjects

The subjects in this study were six healthy volunteers and twenty *P. falciparum* malaria patients (confirmed through microscopic examination) (age range 20–40 years; mean weight 50 kg). Each subject was given two tablets of Quinarsol (each tablet contained 150 mg of quinine; Cipla, Bombay, India) in the morning and evening on day 1 (D1) and on day 2 (D2), with no other drug intake history. Intravenous blood (3 ml) was drawn from each subject after 2.3 h of quinine intake. A 1-ml volume of blood was used for the preparation of a serum sample, and the remaining blood was used for plasma, red blood cells and whole blood (spiked on filter paper) samples. Heparin was used as an anticoagulant wherever required. Capillary blood (100 μl) was also drawn at the same time as intravenous blood, into heparinized precision capillaries, and applied to Whatman No. 1 filter paper. Filter paper samples (100 μl of whole blood) from the venous blood were also prepared using similar precision capillaries. The heparinized blood samples were centrifuged on an IEC Centra-7 centrifuge (International Equipment Company, Needham Heights, MA, USA) for 15 min at 1000 *g* to separate plasma and red blood cells. All samples were kept at 4°C until use.

Extraction

To extract quinine from plasma, serum and red blood cells, 150 μl of 2 *M* sodium hydroxide and 6 ml of enthylene dichloride were added to a 0.5-ml aliquot of the sample. The tubes were shaken for 20 min on an orbital mixer (Denley, Billingshurst, UK) and centrifuged (1000 *g*; 10 min) to separate the phases. The organic phase (4 ml) was taken into a separate test-tube and evaporated to dryness (60°C) on a vortex evaporator (Haake Buchler, Saddle Brook, NJ, USA). To extract quinine from whole blood (filter paper), the paper was cut into small pieces and processed in the same way as other samples. The residue was dis-

solved in 100 μl of the mobile phase, and 25 μl of this solution were used for HPLC analysis.

Calibration

Calibration curves were prepared by analysing 0.5-ml samples of plasma, serum, red blood cells or whole blood (filter paper), spiked with known amounts of quinine. The concentration range of the standards was 0.25–20 μg/ml. Separate standard curves for quinine concentrations expressed as the free base were determined using peak areas.

Recovery and reproducibility

The recovery (extraction yield) was determined at concentrations of 1, 2, 5, 10 and 20 μg quinine per ml of sample by comparing peak areas with areas obtained from direct injection. Within-day reproducibility was evaluated by analysis of samples ($n = 8$) containing 1, 2 and 5 μg of quinine per ml. Day-to-day reproducibility was determined by assaying standard samples and samples from patients. At least fifteen samples each of plasma, serum, red blood cells and whole blood were analysed.

RESULTS AND DISCUSSION

Various proportions of dichloromethane, methanol and perchloric acid were tried to get the separation of quinine, quinidine and other common antimalarials. It was found that the separation of quinine from quinidine was achieved using dichloromethane–methanol–1 *M* perchloric acid (100:9:0.4, v/v). An increase in the proportion of dichloromethane or perchloric acid increased the retention of quinine and quinidine, whereas methanol had the opposite effect. It may be noted that this mobile phase is capable of separating quinine from dihydroquinine, a compound usually found as an impurity in quinine authentic samples.

The capacity factors (k') of most common antimalarial drugs detected by UV (254 nm) are given in Table I, which shows clearly that chloroquine, primaquine, mefloquine, dapsone, amodiaquine, sulfalene, sulfadoxine, sulfamethoxazole, proguanil, pyrimethamine and desethylchloroquine do not interfere in the determination of quinine by this system. Moreover, using fluorescence detection with an excitation wavelength of 350 nm and emission cut-off filters of 418 nm, the peak intensities of most antimalarials are less than 10% of those of quinine and quinidine, thus they do not interfere.

Within-day and day-to-day coefficients of variation (C.V.) averaged 0.74 and 7.56%, respectively (Table II). The average extraction recovery from plasma, serum, red blood cells and whole blood (spiked on filter paper) was 88.1, 87.1, 78.0 and 77.5%, respectively (Table III).

During the study a large number of calibration curves were run. All were linear and the correlation coefficients were always above 0.990. It was also noted that recovery from aqueous solutions was 16.5% higher when the test-tubes were silanized with Aquasil. However, the extraction recovery from any blood medium was not affected

TABLE I

CAPACITY FACTORS OF VARIOUS ANTIMALARIALS

Mobile phase, dichloromethane–methanol–1 *M* perchloric acid (100:9:0.4); flow-rate, 0.8 ml/min; UV detection wavelength, 254 nm; column, Zorbax-Sil (normal phase). k' is a symbol for the capacity factor: $k' = (t_R - t_0)/t_0$, where t_R is retention time of the compound and t_0 is the dead time.

Antimalarial	k'
Quinine	1.49
Dihydroquinine	1.30
Quinidine	1.31
Dihydroquinidine	1.14
Chloroquine	2.57
Mefloquine	0.64
Primaquine	2.85
Amodiaquine	2.91
Sulfalene	0.56
Sulfadoxine	0.68
Dapsone	1.91
Proguanil	1.98
Pyrimethamine	1.18
Sulfamethoxazole	0.80
Desethylchloroquine	3.46

TABLE II

ACCURACY AND PRECISION OF THE HPLC METHOD FOR QUININE IN SPIKED PLASMA SAMPLES

Concentration (μg/ml)	n	C.V. (%)	Accuracy[a] (%)
Within-day			
5	8	0.14	
2	8	0.13	
1	8	1.95	
Mean ± S.D.		0.74 ± 1.04	
Day-to-day			
20	15	8.3	+6.09
10	15	5.2	+9.94
5	15	4.8	−5.9
2	15	9.2	−5.06
1	15	10.3	+6.5
Mean ± S.D.		7.56 ± 2.46	

[a] Accuracy is the deviation from the spiked value.

by silanization. The quinine concentration was determined in all cases using fluorescence detection, because the sensitivity was better than that obtained with UV detection without an internal standard. However, quinine was also determined by using monoethylglycinexylidide (Astra Pharmaceuticals, North Ryde, Australia) as an internal standard with UV detection, and the results were found to be comparable.

Fig. 1 shows the chromatographic behaviour of a blank plasma extract taken from a patient 2.3 h after oral administration of two tablets of Quinarsol. Some endogenous peaks from plasma, serum, red blood cells and whole blood appeared in the chromatogram, but they do not interfere in the determination of quinine.

The plasma quinine concentration profile of six volunteers after oral administration of two tablets of Quinarsol (total 300 mg of quinine) is shown in Fig. 2. The plasma concentration after 4 h of administration of quinine is similar to that reported by Saggers *et al.* [14]. The quinine could be detected for up to four days in plasma.

The average quinine concentrations in plasma, serum and red blood cells of healthy and *P. falciparum* malaria cases after administration of two tablets of Quinarsol in the morning and evening on day 1 (D1) and day 2 (D2) are given in Table IV. It is clear that these average quinine concentrations in *P. falciparum* malaria cases are three to four times greater than those in healthy volunteers at the start of the treatment, and that this factor fell to 1.2 in all blood phases after 32 h of treatment as the parasitaemia declined.

A significant decrease of the volume of distribution of quinine in children suffering from *P. falciparum* malaria compared with uninfected children was reported by Chongsuphajaisiddhi *et al.* [4]. Also, the quinine level in the plasma and

TABLE III

EXTRACTION RECOVERY OF HPLC METHOD FOR QUININE IN PLASMA, SERUM, RED BLOOD CELLS AND WHOLE BLOOD

Concentration (μg/ml)	Recovery (mean + S.D., n = 4) (%)			
	Plasma	Serum	Red blood cells	Whole blood (filter paper)
20	90.95 + 4.6	89.9 ± 3.7	82.3 ± 7	77 ± 10.3
10	89.9 ± 2.7	85.6 ± 2.7	77.0 ± 6.1	78.2 ± 5.9
5	88.3 ± 1.97	86.8 ± 4.4	84.1 ± 4.9	80.9 ± 8.7
2	82.4 ± 3.89	84.9 + 5.2	N.D.[a]	N.D.[a]
1	89.1 + 9.68	88.4 ± 12.5	93.4 ± 8.9	90.0 + 11.8
Mean ± S.D.	88.13 ± 9.12	87.12 ± 10.32	80.0 ± 9.9	77.5 ± 21.2

[a] N.D. = not determined.

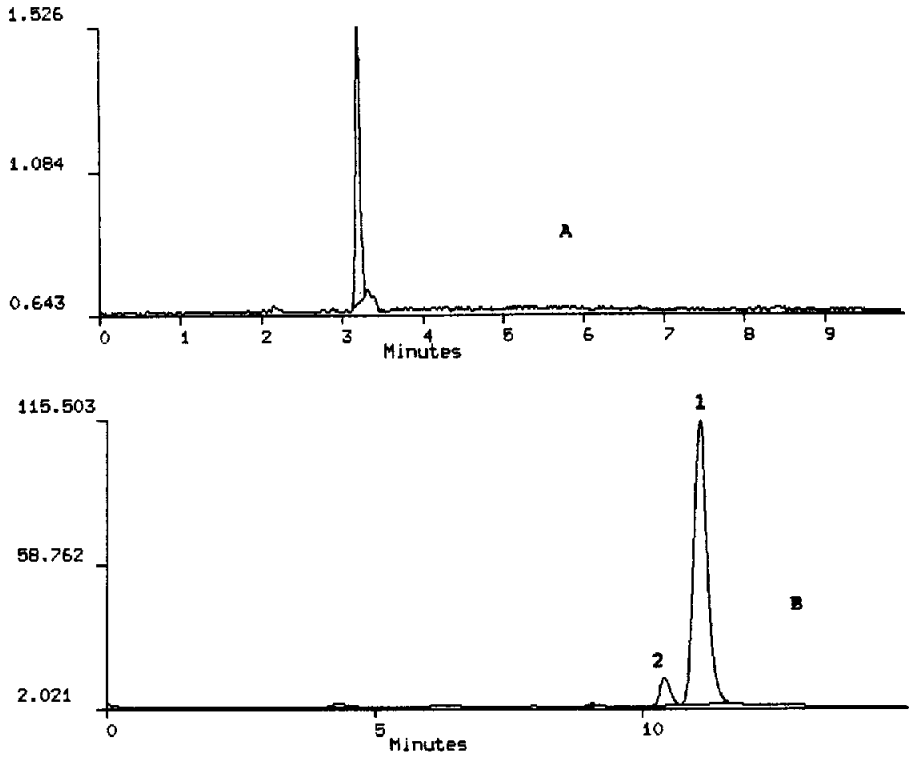

Fig. 1. (A) Chromatogram of a blank plasma extract; flow-rate, 1.2 ml/min. (B) Chromatogram of a plasma extract obtained 2.3 h after oral administration of two tablets of Quinarsol to a *P. falciparum* malaria patient; flow-rate, 0.8 ml/min. Peaks: 1 = quinine (5.0 μg/ml); 2 = dihydroquinine.

serum of healthy people significantly increased with treatment compared with *P. falciparum* malaria cases. However, the quinine level in red blood cells in malaria cases decreased with treatment, which indicated that elimination of quinine from red blood cells in such cases was significantly faster than elimination from plasma or serum. The quinine concentration in red blood cells was found highest in the first few hours of treatment in malaria cases. The ratio of the quinine levels in plasma to red blood cells, or in serum to red blood cells, varies from 3 to 7, which implies that the accumulation of quinine in erythrocytes is much less than that in plasma or serum [15,16].

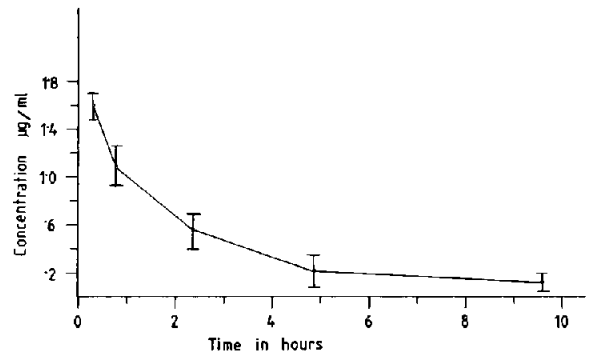

Fig. 2. Average (n = 6) plasma quinine concentration profile after oral administration of two tablets of Quinarsol to healthy people.

The capillary and venous whole blood concentrations of quinine in six volunteers and the *P. falciparum* malaria cases are presented in Table V. It is clear that the concentration of quinine in whole blood drawn from either source is similar in healthy people and malaria patients, which supports the findings of Hellgren *et al.* [12]. However, the average whole blood concentration of quinine in the malaria cases was more than that in the healthy volunteers.

The chromatographic systems described by

TABLE IV

CONCENTRATIONS OF QUININE IN PLASMA, SERUM AND RED BLOOD CELLS IN HEALTHY VOLUNTEERS AND *P. FALCIPARUM* MALARIA CASES

Time after initiation of treatment (h)	Concentration (mean ± S.D.) (μg/ml)					
	Plasma		Serum		Red blood cells	
	Healthy[a]	Malaria[b]	Healthy[a]	Malaria[b]	Healthy[a]	Malaria[b]
2.3	1.60 ± 0.20	5.30 ± 0.60	1.40 ± 0.11	4.60 ± 0.47	0.46 ± 0.22	1.52 ± 0.52
8	3.70 ± 0.42	5.38 ± 0.71	3.42 ± 0.55	4.52 ± 0.70	0.50 ± 0.22	1.24 ± 0.40
18	4.60 ± 0.62	5.62 ± 0.48	3.82 ± 0.38	4.62 ± 0.65	0.52 ± 0.16	1.20 ± 0.38
26	4.20 ± 0.48	5.84 ± 0.29	3.12 ± 0.25	4.92 ± 0.57	0.56 ± 0.18	1.10 ± 0.32
32	5.00 ± 0.64	6.22 ± 0.75	3.62 ± 0.52	4.82 ± 0.48	0.60 ± 0.32	0.84 ± 0.48

[a] Average of six healthy cases.

[b] Average of twenty *P. falciaparum* malaria cases.

Hellgren *et al.* [12] and Zoest *et al.* [11] were not able to separate quinine from quinidine. Recently Mberu *et al.* [13] have reported the separation of quinine from quinidine. However, their method lacks senstivity, and the use of aliphatic amines in the mobile phase may increase the back-pressure in the column. The method described here is highly sensitive with fluorescence detection, for the separation of quinine from dihydroquinine, a compound usually found as an impurity in authentic samples of quinine. It will also separate quinine from quinidine.

Our study clearly shows that there are significant differences in quinine concentrations in plasma, serum and red blood cells of healthy volunteers and *P. falciparum* malaria cases, and that these differences decrease as the patient recovers. This implies that the quinine dose regimen should be adjusted during the convalescence phase. The ratio of the quinine concentrations in plasma and red blood cells varies from 4 to 7, which is in the range of predicted ones [1,2]. The slight difference may be due to the response of the drug to the parasite, the bioavailability of the drug, or the selectivity and specificity of the HPLC method.

TABLE V

CONCENTRATION OF QUININE IN WHOLE BLOOD

Time after initiation of treatment (h)	Concentration (mean ± S.D.) (μg/ml)			
	Healthy cases (n = 6)		*P. falciparum* malaria cases (n = 10)	
	Capillary blood	Venous blood	Capillary blood	Venous blood
2.3	0.56 ± 0.14	0.60 ± 0.13	1.66 ± 0.90	1.50 ± 0.86
8.0	1.38 ± 0.42	1.4 ± 0.43	2.10 ± 0.72	2.20 ± 0.58

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