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High-performance liquid chromatographic determination of primaquine and carboxyprimaquine concentrations in plasma and blood cells in *Plasmodium vivax* malaria cases following chronic dosage with primaquine

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Abstract

A reversed-phase HPLC method using acetonitrile–methanol–1 *M* perchloric acid–water (30:9:1:95, v/v) at a flow-rate of 1.5 ml/min on a μ-Bondapak C_{18} column with UV detection at 254 nm was developed for the separation of primaquine, its major metabolite carboxyprimaquine and other metabolites such as N-acetylprimaquine, 4-hydroxyprimaquine, 5-hydroxyprimaquine, 5-hydroxy-6-methoxyprimaquine, demethylprimaquine and 6-methoxyprimaquine, and also other antimalarials. The calibration graphs were linear in the range 0.025–100 μg/ml for primaquine and 4–1000 μg/ml for carboxyprimaquine. The within-day and day-to-day coefficients of variation averaged 3.65 and 6.95%, respectively, for primaquine and 3.0 and 7.52%, respectively, for carboxyprimaquine in plasma. The extraction recoveries for primaquine and carboxyprimaquine were 89 and 83%, respectively. The mean carboxyprimaquine concentration was much higher in plasma and blood cells of *Plasmodium vivax* patients than that in plasma from healthy subjects. The carboxyprimaquine level was also higher in blood cells than plasma whereas the primaquine concentration was the same in both cases.

1. Introduction

Primaquine is important because of its activity against several life-cycle stages of the malaria parasite [1]. Its practical use, however, is associated with significant toxicity, especially in persons deficient in glucose-6-phosphate dehydrogenase (G6PD). HPLC methods have been reported for the determination of primaquine and its metabolites in biological fluids [2–4] but all are used for healthy volunteers. Bhatia et al. [5] reported pharmacokinetic studies of primaquine in whole blood with *Plasmodium vivax* malaria cases using HPLC and Ward et al. [6] compared the pharmacokinetic parameters of primaquine in healthy Thai subjects with samples from acute and chronic dosages. Recently, Edwards et al. [7] evaluated the pharmacokinetic properties of primaquine in healthy volunteers given mefloquine orally and in patients treated with quinine followed by primaquine for acute falciparum malaria, while Bangchang et al. [8] determined

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primaquine concentrations on G6PD-deficient and G6PD-normal patients with vivax malaria. Fletcher et al. [9] found that erythrocytes play a major role in the distribution of the drug and its metabolites during a study on the biochemical pharmacology of primaquine in Rhesus monkeys and rats.

We report here a reversed-phase HPLC method for the separation of primaquine and its metabolite carboxyprimaquine in plasma and blood cells and the determination of their concentrations in plasma and blood cells of *Plasmodium vivax* malaria cases following the regimen of chloroquine with a 5-day chronic dosage of primaquine.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol were of HPLC grade and *n*-hexane, ethyl acetate and all other chemicals were of analytical-reagent grade and were used without further purification.

Primaquine diphosphate was purchased from Sigma (St. Louis, MO, USA) and carboxyprimaquine and N-acetylprimaquine were received as a kind gift from Professor McChesney, University of Mississippi, USA. 4-Hydroxyprimaquine, 5-hydroxyprimaquine, 6-hydroxyprimaquine, 5-hydroxy-6-methoxyprimaquine, demethylprimaquine and 6-methoxyprimaquine (internal standard) were provided by the Department of Medicinal Chemistry, Department of Experimental Therapeutics, Walter Reed Army Institute of Research (Washington, DC, USA). Primaquine tablets (each containing 7.5 mg of primaquine base) were supplied by the National Malaria Eradication Programme (NMEP), India.

2.2. Preparation of standard solutions

Stock standard solutions of primaquine (100 μg/ml) and carboxyprimaquine (1500 μg/ml) were prepared in methanol. Intermediate working standard solutions covering the concentration range 4–1000 μg/ml as reported by Ward et al. [6] were prepared by diluting the stock standard solution with methanol. All solutions were stored at 4°C.

2.3. Chromatography

Chromatography was performed on a DuPont (Wilmington, DE, USA) HPLC system consisting of a Model 8800 pump module, a Rheodyne injector, a variable-wavelength UV detector operated at 254 nm and an integrator. The mobile phase was acetonitrile–methanol–1 *M* perchloric acid–water (30:9:1:95, v/v), pumped at a flow-rate of 1.5 ml/min through a μBondapak C_{18} reversed-phase column (300 × 3.9 mm I.D.; 5 μm particle size). The mobile phase was filtered and degassed by ultrasonication (FS 100; Decon, Hove, UK) before use. Chromatography was performed at ambient temperature.

2.4. Subjects

The subjects were 51 *Plasmodium vivax* malaria cases (confirmed through microscopic examination), age group 14–52 years, mean body mass 63.4 kg, from Indian Oil (Mathura, U.P., India). After ascertaining that no antimalarial had been taken before starting the treatment, all patients were given 900 mg of chloroquine base (600 on day 1, 300 on day 2) and 75 mg of primaquine base (15 mg of primaquine per day for 5 days, i.e., chronic dosage).

2.5. Preparation of plasma and blood cells

Intravenous blood (2.5 ml) was drawn from patients on days 1, 2, 3, 4 and 5 2.5 h post-dose. Samples were taken in such a way that a maximum of two samples were taken from each patient to avoid practical problems. Heparin was used as an anticoagulant. The heparinized blood samples were centrifuged on IEC Centra-7 centrifuge (International Equipment, Needham Heights, MA, USA) for 15 min at 1000 *g* to separate plasma and blood cells. It may be noted that the blood cells consisted of red blood cells, thrombocytes and leukocytes. All samples were kept at 4°C until used.

2.6. Extraction

For the extraction of primaquine and its metabolite carboxyprimaquine from plasma and blood cells, the procedure of Ward et al. [2] was followed with some modifications. Briefly, to 0.5 ml of the sample (standard or analysis), 100 μl of internal standard (0.05 μg per 100 μl) and 2 ml of 25% ammonia solution (specific gravity 0.91) were added and vortex mixed for 2 min. The mixture was extracted with *n*-hexane–ethyl acetate (3.5:0.5, v/v) and centrifuged at 1000 *g* for 10 min to separate the phases. The organic phase was separated and evaporated to dryness at 60°C on a Haake Buchler (Saddle Brook, NJ, USA) vortex evaporator. The residue was reconstituted with 100–250 μl of mobile phase and 20–250 μl of this solution were injected for HPLC analysis.

2.7. Calibration procedure

Calibration graphs were prepared by analysing 0.5-ml samples of plasma or blood cells spiked with known amounts of primaquine and carboxyprimaquine. The concentration range of primaquine was 0.025–100 μg/ml and that of carboxyprimaquine was 4–1000 μg/ml.

2.8. Accuracy and intra- and inter-assay precision

The within-day reproducibility (coefficient of variation, C.V.) was evaluated by the analysis of five samples, each containing 0.025 and 0.100 μg/ml of primaquine and 4.0 and 32.0 μg/ml of carboxyprimaquine. The day-to-day reproducibility was determined by assaying standard samples of different concentrations and the accuracy was determined by analysing samples of known concentration ($n = 5$). At least five samples each of plasma and blood cells were analysed.

2.9. Recovery

The recovery (extraction yield) was determined at primaquine concentrations in the samples of 0.025, 0.05 and 0.15 μg/ml and carboxyprimaquine concentrations of 4.0, 16.0 and 32.0 μg/ml by comparing the peak areas with the areas obtained with direct injection.

3. Results and discussion

The mobile phase consisting of acetonitrile–methanol–1 *M* perchloric acid–water (30:9:1:95, v/v) at a flow-rate of 1.5 ml/min was found to be the most suitable for achieving the separation of primaquine, carboxyprimaquine, N-acetylprimaquine, 4-hydroxyprimaquine, 5-hydroxyprimaquine, 6-hydroxyprimaquine, 5-hydroxy-6-methoxyprimaquine, demethylprimaquine and 6-methoxyprimaquine (internal standard) in addition to their separation from other common antimalarials such as chloroquine, sulfalene, quinine, sulfadoxine, pyrimethanine and dapsone. The retention factors (k) of primaquine, its derivatives and other common antimalarials are given in Table 1, which clearly shows that chloroquine, sulfalene, sulfadoxine, pyrimethamine, dapsone and quinine do not interfere in the detection of primaquine and its metabolite carboxyprimaquine by this method. Moreover, the method is also suitable for the identification of primaquine derivatives such as 4-hydroxyprimaquine, 5-hydroxyprimaquine, 6-hydroxyprimaquine, 5-hydroxy-6-methoxyprimaquine, demethylprimaquine and N-acetylpyrimaquine (Fig. 1).

An increase in the proportion of acetonitrile in the mobile phase decreases the retention whereas an increase in water or perchloric acid content increases the retention of primaquine and its metabolites, thereby following a reversed-phase and ion-pair mechanism as stated earlier [10]. During the study, a large number of calibration graphs were obtained and all were linear with correlation coefficients above 0.98. The limit of quantification was 10 ng/ml. The within-day and day-to-day coefficients of variation (C.V.) were 3.65 and 6.95% for primaquine and 3.0 and 7.52% for carboxyprimaquine, respectively (Table 2). The average recoveries of primaquine and carboxyprimaquine were 89 and 83.40%, respectively.

Fig. 2A shows the chromatographic behaviour

Table 1
Retention factors (k) of different antimalarials

Antimalarial	k^a	Antimalarial	k^a
Primaquine (PQ)	2.01	PQ-carboxylic acid	4.19
N-Acetyl-PQ	3.76	Chloroquine	1.82
4-Hydroxy-PQ	0.88	Sulfadoxine	1.62
5-Hydroxy-PQ	0.74	Sulfalene	1.37
6-Hydroxy-PQ	0.21	Pyrimethamine	3.78
5-Hydroxy-6-methoxy-PQ	0.76	Dapsone	1.33
Demethyl-PQ	0.42	Quinine	0.81
6-Methoxy-PQ	1.02		

Mobile phase, acetonitrile–methanol–1 M perchloric acid–water (30:9:1:95, v/v); flow-rate, 1.5 ml/min; UV detection at 254 nm; column, μBondapak C_{18}.
[a] $k = (t_R - t_0)/t_0$, where t_R is the retention time of the compound and t_0 is the dead time.

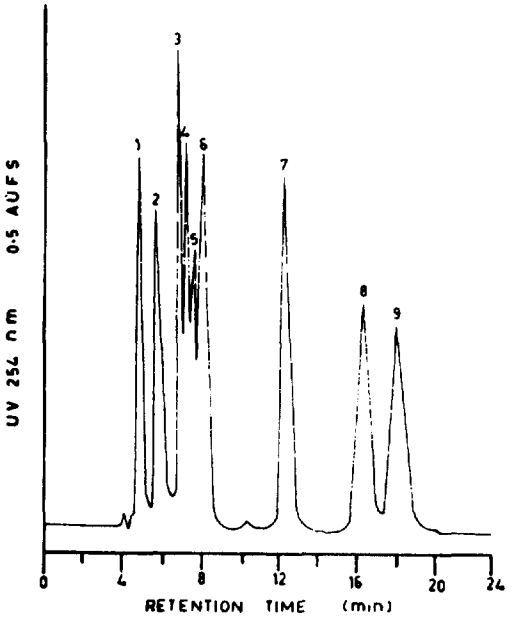

Fig. 1. Chromatogram showing the separation of primaquine and its derivatives on a μBondapak C_{18} column using acetonitrile–methanol–1 M perchloric acid–water (30:9:1:95, v/v) as the mobile phase at a flow-rate of 1.5 ml/min with UV detection at 254 nm. Peaks: 1 = 6-hydroxyprimaquine (0.05 μg/ml); 2 = demethylprimaquine (0.04 μg/ml); 3 = 5-hydroxyprimaquine (0.05 μg/ml); 4 = 5-hydroxy-6-methoxyprimaquine (0.05 μg/ml); 5 = 4-hydroxyprimaquine (0.05 μg/ml); 6 = 6-methoxyprimaquine (0.05 μg/ml); 7 = primaquine (0.05 μg/ml); 8 = N-acetylprimaquine (0.03 μg/ml); 9 = carboxyprimaquine (8 μg/ml).

Table 2
Accuracy and precision of HPLC method for primaquine and its metabolite carboxyprimaquine (spiked plasma samples; $n = 5$)

Concentration (μg/ml)	C.V. (%)		Accuracy (%)
	Within-day	Day-to-day	
Primaquine			
0.100	2.8	5.2	5.86
0.025	4.5	8.7	2.00
Mean ± S.D.	3.65 ± 1.20	6.95 ± 2.47	
Carboxyprimaquine			
32.0	3.1	6.82	4.55
4.0	2.9	8.22	1.50
Mean ± S.D.	3.0 ± 0.14	7.52 ± 0.98	

of a blank plasma extract from a healthy volunteer before administration of the drug and a plasma extract of a *Plasmodium vivax*-infected patient on day 3 after 2.3 h following oral administration of primaquine at 15 mg per day for 5 days. The chromatographic behaviour of extracts from plasma and blood cells was similar. Some endogenous peaks from plasma and blood cells appeared in the chromatogram but they did not interfere in the determination of primaquine and carboxyprimaquine by this method (Fig. 2B).

The mean primaquine and carboxyprimaquine concentrations in plasma and blood cells of *Plasmodium vivax*-infected malaria cases following oral administration of 15 mg of primaquine

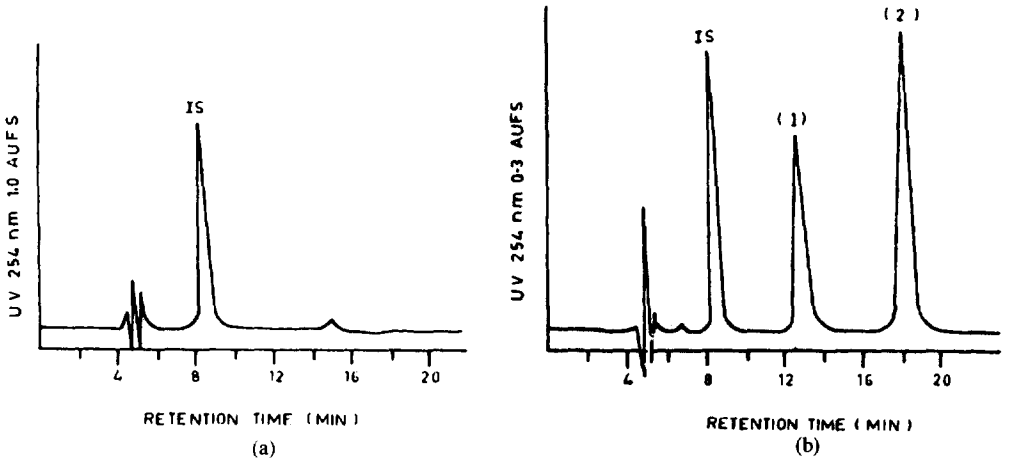

Fig. 2. Chromatograms showing separations of primaquine and its metabolite on a μBondapak C_{18} column with acetonitrile–methanol–1 *M* perchloric acid–water (30:9:1:95, v/v) as the mobile phase at a flow-rate of 1.5 ml/min with UV detection at 254 nm. (A) Chromatogram of a blank plasma extract containing 6-methoxyprimaquine [internal standard (I.S.), 0.05 μg/ml] obtained from a volunteer; (B) chromatogram of a plasma extract taken 2.5 h post-dose on day 3 after oral administration of 15 mg of primaquine to a *P. vivax* patient. Peaks: 1 = primaquine (0.05 μg/ml); 2 = carboxyprimaquine (8 μg/ml).

per day for 5 days are given in Table 3. It is clear that the mean primaquine concentrations in plasma and blood cells were same for 5 days in spite of the chronic dosage. Similar primaquine behaviour has been reported with Thai healthy volunteers [6] and also for *P. vivax* malaria cases [5,8]. However, studies in monkeys have indicated that primaquine and its metabolites were concentrated in red blood cells [11]. Singhasivanon et al. [12] could not detect primaquine in red blood cells of healthy volunteers. It was also observed that the carboxyprimaquine level increased after chronic dosage whereas primaquine remained at the same level in plasma and blood cells. Moreover, the carboxyprimaquine concentrations were found to be

Table 3
Mean (±S.D.) plasma and blood cell concentrations[a] of primaquine (PQ) and carboxyprimaquine (CPQ) in *P. vivax* cases following administration of primaquine (base) at 15 mg/day for 5 days

Day	Concentration[b] (μg/ml)			
	Plasma		Blood cells	
	PQ	CPQ	PQ	CPQ
1	0.061 ± 0.015	2.35 ± 1.11	0.057 ± 0.0147	10.07 ± 7.21
	(0.037–0.084)	(1.0–4.4)	(0.038–0.079)	(3.28–22.21)
2	0.062 ± 0.022	5.64 ± 3.72	0.057 ± 0.020	64.90 ± 48.11
	(0.040–0.096)	(1.55–13.74)	(0.03–0.089)	(13.0–146.87)
3	0.061 ± 0.014	8.19 ± 4.50	0.056 ± 0.015	129.91 ± 153.20
	(0.036–0.085)	(2.21–15.0)	(0.033–0.079)	(27.32–547.93)
4	0.063 ± 0.018	15.03 ± 12.63	0.055 ± 0.019	261.55 ± 210.97
	(0.038–0.084)	(2.50–47.30)	(0.036–0.092)	(28.35–692.0)
5	0.062 ± 0.014	16.28 ± 11.71	0.055 ± 0.033	548.02 ± 225.49
	(0.041–0.083)	(6.03–32.0)	(0.033–0.060)	(287.62–969.42)

[a] Mean concentrations of ten cases per day.
[b] Values in parentheses indicate concentration range.

many times higher than those of primaquine, which may be due to the rapid metabolism of primaquine into carboxyprimaquine [13]. The inter-individual difference was threefold for primaquine in both plasma and blood cells, which is low compared with earlier reports for healthy volunteers [6], and the inter-individual difference for carboxyprimaquine was 4–18-fold for plasma and 6–25-fold for blood cells. These large inter-subject variations suggest variable bioavailability and rapid metabolism [13]. The concentration of carboxyprimaquine was many times higher in blood cells than plasma, supporting Fletcher et al.'s view [9] that erythrocytes play a major role in the distribution and metabolism of the parent drug and its metabolites.

Baker et al. [11] found that primaquine and its metabolites concentrated in red blood cells of monkeys. From day 1 to day 5 the ratio of carboxyprimaquine to primaquine increased from 38 to 262 in plasma and from 136 to 9964 in blood cells after chronic dosage. Ward et al. [6] found a ratio of 10 in plasma of healthy Thai subjects and Breckenridge et al. [13] reported a ratio of 50 in plasma of healthy volunteers. A much higher ratio of plasma of *P. vivax* cases as compared with healthy subjects implies that the primaquine metabolized much faster into carboxyprimaquine in *P. vivax* malaria cases than in healthy subjects. Bhatia et al. [5] found that the carboxyprimaquine to primaquine ratio varied from 74 to 143 in whole blood of *P. vivax* malaria cases.

The chromatographic system described here is suitable for the separation and identification of primaquine and its derivatives. The method is sensitive and selective also for the determination of primaquine and carboxyprimaquine in plasma and blood cells. The substitution of an N-acetyl or carboxy group in primaquine increases the retention whereas a hydroxyl group decreases the retention, thereby following reversed-phase behaviour. Further, the separation of primaquine from other antimalarials such as chloroquine, sulfadoxine, sulfalene, dapsone and quinine implies that the separation was based on a reversed-phase and ion-pair mechanism.

This study has clearly shown very high concentrations of carboxyprimaquine in plasma and blood cells of *P. vivax* malaria cases compared with earlier reports for healthy subjects, which implies the rapid metabolism of primaquine into carboxyprimaquine in *P. vivax* cases. The study has also shown low accumulations of primaquine in blood cells very similar to those in plasma, but a higher concentration of carboxyprimaquine in blood cells than plasma, which may have some therapeutic significance for the treatment of *P. vivax* malaria cases.

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