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Short communication

Sulfalene concentrations in plasma and blood cells of *Plasmodium falciparum* malaria cases after treatment with metakelfin using high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatographic method using acetonitrile–methanol–1 *M* perchloric acid–water (25:9:0.8:95, v/v/v) at a flow-rate of 1.0 ml min^{-1} on LiChrospher 100 RP 18 column (250×4 mm; 5 μm) with UV (254 nm) detection has been developed for the determination of sulfalene in plasma and blood cells after oral administration of the antimalarial drug metakelfin. Calibration curves were linear in the range 0.5–100 μg ml^{-1}. The limit of quantification was 50 ng ml^{-1}. Within-day and day-to-day coefficients of variation averaged 3.84 and 5.31%, respectively. Mean extraction recoveries of sulfalene from plasma and blood cells were 87.21 and 84.65%, respectively. Mean concentrations of sulfalene in plasma of *P. falciparum* cases on days 2, 7 and 15 were 44.58, 14.90 and 1.70 μg ml^{-1}, respectively; in blood cells concentrations of sulfalene were 7.77, 3.25 and 0.75 μg ml^{-1}, respectively, after oral treatment with two tablets (1000 mg) of metakelfin. Significant difference was recorded on day 2 for sulfalene concentration in blood cells of healthy and *P. falciparum* cases ($t=9.49$; $P<0.001$).

Keywords: Sulfalene; Metakelfin

1. Introduction

The emergence of *Plasmodium falciparum* resistance to many antimalarial drugs is becoming a severe problem all over the world. Metakelfin (500 mg of sulfalene+25 mg of pyrimethamine) is frequently used for prophylaxis and treatment of malaria where chloroquine resistance is present [1]. Very little information is available on sulfalene concentrations in different body fluids, and that is limited to healthy cases. Recently, a normal-phase high-performance liquid chromatographic (HPLC) method was developed for the determination of sulfalene in plasma, whole blood and red blood cells [2]. We now describe a reversed-phase HPLC method for the determination of sulfalene concentration in plasma and blood cells of *P. falciparum* malaria cases after oral administration of two tablets of metakelfin.

2. Experimental

The study was conducted in the Shankargarh block known as the stone quarry area of Allahabad district

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(Uttar Pradesh, India). This region has been characterized by a high incidence of malaria, particularly of *P. falciparum*, for several years. Chloroquine resistance has already been reported from this block as well as from adjacent districts but chloroquine resistant cases have responded to a combination of sulfalene and pyrimethamine [3].

2.1. Chemicals and standards

HPLC grade acetonitrile, methanol and ethylene dichloride were obtained from Spectrochem (Mumbai, India). All other reagents were of analytical grade and were used without further purification. Sulfalene and sulfamethoxazole were supplied by ICI (Australia) and their structures are given in Fig. 1. Sulfamethoxazole was used as an internal standard (I.S.). Stock solutions of sulfalene and sulfamethoxazole (5 mg ml^{-1} each) were prepared separately in methanol. Intermediate and working standard solutions covering the concentration range 0.5–100 μg ml^{-1} were prepared by diluting the stock standard solution with methanol. All solutions were stored at 4°C. A 12 m*M* phosphate buffer solution (0.2738 g of $K_2HPO_4 \cdot 3H_2O$ in 100 ml distilled water, pH 3.40) was prepared by adding 0.1 ml of acetic acid to 9.9 ml of phosphate buffer.

2.2. Subjects

The subjects in this study were 50 *P. falciparum*-infected malaria cases (33 male and 17 female; age range 20–60 years; mean weight 45.68 kg) confirmed through microscopic examination. They had no known allergy to sulfonamides and had not taken any other antimalarial drug before treatment. Each subject was given two tablets of metakelfin (Dominion, Calcutta, India) consisting of a total of 1000 mg sulfalene+50 mg of pyrimethamine as per National Drug Policy. The patients were supervised while swallowing and for 30 min afterwards. Blood smears were collected on day 0 (D0), 2 (D2), 7 (D7) and 15 (D15). Asexual parasites were examined from Giemsa's stained smears. Intravenous blood (3.0 ml) was drawn from each subject on D2, D7 and D15. EDTA was added as an anticoagulant. Blood was centrifuged for 15 min at 1000 *g* to separate plasma and blood cells. All samples were stored at 4°C until analysis.

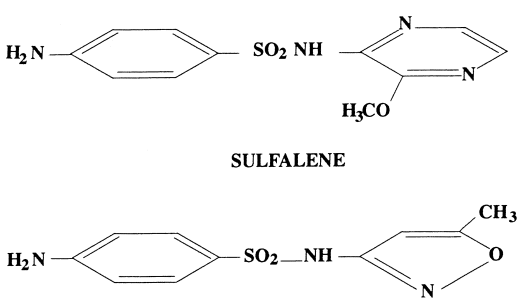

Fig. 1. Structures of sulfalene and sulfamethoxazole (I.S.).

2.3. Instrumentation and chromatographic conditions

Chromatography was performed on a Waters HPLC system (Waters, Milford, MA, USA) consisting of a 510 pump, a 486 UV detector operated at 254 nm, a Rheodyne injector and a Spectra-Physics integrator (San Jose, CA, USA). The mobile phase was acetonitrile–methanol–1 *M* perchloric acid–water (25:9:0.8:95, v/v/v) pumped at a flow-rate of 1.0 ml min^{-1} through a LiChrospher RP 18 column (250×4 mm, 5 μm). The mobile phase was filtered and degassed by ultrasonication (FS 100; Decon Hove, UK) before use. Chromatography was performed at ambient temperature.

2.4. Extraction

Extraction of sulfalene from plasma and blood cells was performed as described elsewhere [2,4]. Briefly, to an aliquot of 0.5 ml of sample (standard or analyse) were added 0.5 ml of distilled water, 100 μl of phosphate buffer (pH 3.40) and 6 ml of ethylene dichloride. The test tube was shaken for 20 min on a Denley orbital mixer (Billingshurst, UK) and centrifuged at 1000 *g* for 15 min to separate the phases. The organic phase was transferred to a clean glass tube and evaporated to dryness at 60°C on a Haake Buchler vortex evaporator (Saddle Brook, NJ, USA). The residue was dissolved in 100 μl of the mobile phase and 10–30 μl of this solution was injected for HPLC analysis.

2.5. Recovery and reproducibility

The recovery was determined at concentrations of 2.5, 5.0, 10, 20, 30 and 40 μg of sulfalene per ml of plasma/blood cells by comparing peak-height ratios of spiked standards with ratios obtained by direct injection of pure standards. Within-day and day-to-day reproducibility of the method were determined by repeated assay of different concentrations of the sulfalene.

2.6. Stability of sulfalene

The stability of sulfalene was determined in the samples stored over a period of 3 months at 4°C and their concentrations were determined at regular intervals.

3. Results and discussion

Various proportions of acetonitrile, methanol, perchloric acid and water as mobile phase were used to achieve the separation of sulfalene, sulfamethoxazole and pyrimethamine. It was found that the separation of sulfalene and sulfamethoxazole was achieved using the chromatographic method described above. Pyrimethamine eluted after sulfalene and sulfamethoxazole and thus did not interfere with the separation. Capacity factors (k') of sulfalene, sulfamethoxazole and pyrimethamine are 2.39, 3.53 and 8.18, respectively. The behaviour of sulfalene and sulfamethoxazole on changing the mobile phase composition was found to be similar to that reported earlier [5] for sulfadoxine. Briefly, an increase in the proportion of acetonitrile decreased the retention while an increase in perchloric acid or water increased the retention of sulfalene. The reversed-phase HPLC method described above was found to be better than the earlier normal-phase HPLC method [2] because of solvent stability and column life. Fig. 2 shows the chromatograms of a blank plasma extract from a healthy volunteer and the plasma extract of a patient on D2 after oral administration of two tablets of metakelfin. Chromatographic behaviour of extracts from blood cells and plasma was similar.

During the study, a large number of calibration curves was obtained for the concentration range of 0.5–100 μg ml^{-1}. All were linear and the correlation coefficients were always above 0.99.

No degradation was detected for sulfalene storage in plasma or blood cells at 4°C for over 3 months. The limit of detection for sulfalene was 5 ng with a signal-to-noise ratio of 3:1 using the prescribed method while the limit of quantification was 50 ng ml^{-1}. Within-day and day-to-day coefficients of variations (C.V.) averaged 3.84 and 5.31%, respectively (Table 1). Mean extraction recoveries of sulfalene in plasma and blood cells were 87.21 and 84.65%, respectively (Table 2) and were similar to those reported earlier [2].

All 50 *P. falciparum* cases responded well to metakelfin treatment and the parasite cleared within 3 days of treatment except in two cases where it cleared within 7 days. The mean parasite density on D0 was 5210 mm^{-3} (range 600–21000 mm^{-3}). The mean concentration profile of sulfalene in plasma and blood cells of 50 *P. falciparum* cases under study on D2, D7 and D15 after administration of two tablets of metakelfin as a therapeutic dose are given in Table 3. The mean sulfalene concentration in plasma was high compared to the concentration in blood cells. This may be due to high protein binding of sulfalene, mainly to albumin, due to its acidic character, and therefore it does not accumulate much in the erythrocytes [6]. The inter-individual variations of sulfalene concentrations in different blood components were between 2- and 4-fold, which is similar to the variations reported for sulfadoxine [5].

A comparison of sulfalene concentrations in blood cells of *P. falciparum* cases with sulfalene in healthy cases reported earlier [2] showed that the concentration in blood cells of *P. falciparum* cases was low compared to healthy volunteers, particularly within the first few days of treatment; a statistically significant difference was recorded on D2 ($t=9.49$; $P<0.001$). The low concentration of sulfalene in blood cells of *P. falciparum* cases may be due to a low level of haemoglobin because sulfonamides present in erythrocytes are partially bound to haemoglobin [6]. This is further supported by the fact that the difference in sulfalene concentrations between healthy and *P. falciparum* cases was not significant on D7 and D15 because the haemoglobin level increased as the patient recovered from malaria. How-

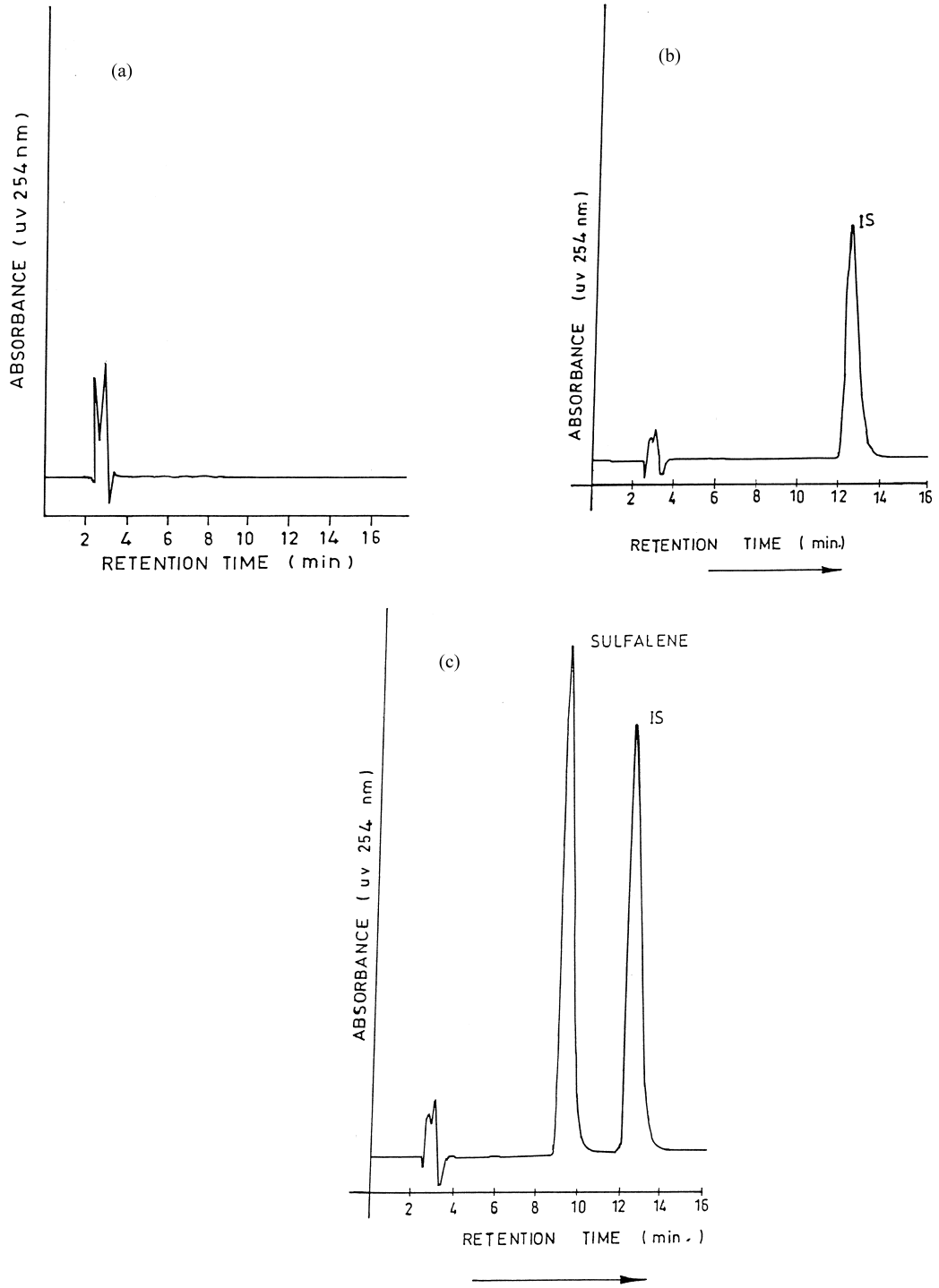

Fig. 2. Chromatogram of (a) blank plasma extract, (b) blank plasma extract containing sulphamethoxazole (I.S.) obtained from a volunteer before a drug administration and (c) a plasma extract taken on D2 after oral administration of two tablets of metakelfin.

Table 1
Precision of the HPLC method for sulfalene in plasma (spiked samples)

	Concentration ($\mu g\ ml^{-1}$)	n	C.V. (%)
Within-day	20	4	2.47
	40	4	5.21
Mean±S.D.	3.84±1.93		
Day-to-day	2.5	5	7.08
	5.0	5	5.89
	10.0	5	5.77
	20.0	5	4.37
	30.0	5	4.43
	40.0	5	4.34
Mean±S.D.	5.31±1.12		

n=Number of observations.

Table 2
Extraction recovery of the HPLC method for sulfalene in plasma and blood cells

Concentration ($\mu g\ ml^{-1}$)	Recovery (%) (mean±S.D., n=4)	
	Plasma	Blood cells
2.5	85.63±7.6	75.88±2.39
5.0	85.82±1.86	83.39±7.22
10.0	88.97±6.51	89.09±1.90
20.0	85.30±9.67	84.78±6.22
30.0	89.41±4.28	87.64±1.12
40.0	88.18±1.15	87.12±7.45
Mean±S.D.	87.21±1.84	84.65±4.75

n=Number of observations.

Table 3
Sulfalene concentration in plasma and blood cells of *P. falciparum* cases after treatment with two tablets of metakelfin

	Concentration[a] (mean±S.D.) ($\mu g\ ml^{-1}$)		
	Day 2	Day 7	Day 15
Plasma	44.58±12.06 (22.95–69.15)	14.90±3.99 (7.95–23.32)	1.70±0.62 (0.90–2.92)
Blood cells	7.77±3.00 (3.75–12.97)	3.25±1.25 (1.57–5.17)	0.75±0.10 (0.63–0.82)

[a] Concentrations are an average of 50 *P. falciparum* patients. Concentration range in parenthesis.

ever, the difference in plasma sulfalene concentrations of *P. falciparum* and healthy cases was found to be insignificant.

The average concentration ratio of sulfalene in blood cells to plasma on D2, D7 and D15 in *P. falciparum* cases were 0.17, 0.21 and 0.16, respectively, which were low compared to the ratio reported for healthy cases [2]. Low ratios in *P. falciparum* cases are due to low uptake of sulfalene in parasitized red blood cells because of anaemia, defective red cell production or by impaired release of red blood cells in the circulation in *P. falciparum* malaria cases [7,8].

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