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Sulphadoxine concentrations in plasma, red blood cells and whole blood in healthy and *Plasmodium falciparum* malaria cases after treatment with Fansidar using high-performance liquid chromatography

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Abstract: A reversed-phase high-performance liquid chromatographic method using acetonitrile–methanol–(1M) perchloric acid–water (30:9:0.8:95, v/v/v/v) at a flow of 1.5 ml min^{-1} on μ-Bondapak C_{18} column with UV (254 nm) detection has been developed for the separation of sulphadoxine, sulphalene and sulphamethoxazole from other antimalarials. Calibration curves were linear in the range 0.5–100 μg ml^{-1}. The limit of quantitation was 50 ng ml^{-1}. Within-day and day-to-day coefficients of variation averaged 2.1 and 6.45%, respectively. The extraction recovery of sulphadoxine from plasma, red blood cells and whole blood was 90.28, 92.05 and 94.69%, respectively. The method has been used for the determination of sulphadoxine concentrations in plasma, red blood cells and whole blood of eight healthy and 50 *Plasmodium falciparum* malaria cases after administration of two tablets of Fansidar. Mean sulphadoxine concentration in plasma was higher than red blood cells or whole blood. Sulphadoxine concentration in plasma and whole blood of *P. falciparum* malaria cases was significantly higher as compared to healthy volunteers while it was the same in red blood cells. Sulphadoxine was absorbed much less in red blood cells than in plasma or whole blood.

Keywords: *Reversed-phase chromatography; sulphadoxine;* Plasmodium falciparum *malaria cases.*

Introduction

Increasing resistance of *Plasmodium falciparum* to 4-aminoquinoline drugs is a major world health problem. Fansidar (500 mg sulphadoxine and 25 mg pyrimethamine) is frequently used for the prophylaxis and treatment of malaria in areas with chloroquine resistant strains [1]. Severe cutaneous adverse reactions to Fansidar during prophylaxis are well known [2, 3]. Determination of drug concentrations in different body fluids is important for proper prophylaxis and treatment of malaria cases, as well as in the investigation of dose-dependent adverse reactions. Spectrophotometric methods [4] were used for the analysis of sulphadoxine until the late 1960s. High-performance liquid chromatographic (HPLC) methods have been applied now for the assay of sulphadoxine in plasma [5, 6, 7], serum [8] and whole blood [9] of healthy volunteers. Sarikabuthi *et al.* [10] determined plasma concentrations of sulphadoxine in healthy and *Plasmodium falciparum* cases in Thai patients using spectrophotometric method while Hellgren *et al.* [11] have reported sulphadoxine whole blood concentrations in Tanzanian school children with *P. falciparum* malaria using a HPLC method. A review of the literature [12] reveals that studies on concentrations of sulphadoxine in different body fluids in *P. falciparum* malaria cases and their difference from healthy volunteers using a sensitive HPLC method are lacking. We describe a reversed-phase HPLC method for the determination of sulphadoxine concentrations in plasma, red blood cells and whole blood and a comparison of the concentrations in healthy and *P. falciparum* malaria cases after administration of two tablets of Fansidar.

Materials and Methods

Chemicals and standards

HPLC grade acetonitrile, methanol and ethylene dichloride were obtained from Sisco

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Research Labs (Bombay, India). All other reagents were of analytical reagent grade and were used without further purification.

Sulphadoxine and sulphamethoxazole were supplied by Roche Products Ltd (Bombay). Sulphamethoxazole was used as an internal standard. Stock standard solutions of sulphadoxine and sulphamethoxazole (5 mg ml^{-1} each) were prepared separately in methanol. Intermediate and working standard solutions covering the concentration range reported by Edstein [5] were prepared by diluting the stock standard solution with methanol. All solutions were stored at 4°C.

A 12 mM phosphate buffer solution (pH 3.40) was prepared by adding 0.1 ml of acetic acid to 9.9 ml of phosphate buffer.

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 with a sensitivity setting of 1.0 a.u.f.s., a WISP 712 automatic sample injector and a Spectra–Physics integrator was used for analysis. The column was a (3.9 × 300 mm i.d. particle size 10 μm), μ-Bondapak C_{18} (Waters Assoc.) reversed-phase column.

The mobile phase consisting of acetonitrile, methanol, (1 M) perchloric acid and water (30:9:0.8:95, v/v/v/v) was pumped at a flow rate of 1.5 ml min^{-1} at ambient temperature. The mobile phase was filtered and degassed by ultrasonication (Decon FS100, Hove, UK) before use.

Subjects

The subjects in this study were eight healthy volunteers (five male and three female; age range 25–35 years; mean wt 42 kg) and 50 *P. falciparum* malaria patients (confirmed through microscopic examination) (33 male and 17 female; age range 20–60 years; mean wt 42.68 kg). Two tablets of Fanisdar (each consisting of 500 mg sulphadoxine and 25 mg pyrimethamine) were given to each subject as per National drug policy [13] with no other drug intake history and without known allergy against sulphonamides. Intravenous blood (2.5 ml) was drawn from each subject on Day 2 (D2), Day 7 (D7) and Day 15 (D15) out of which 0.5 ml whole blood was taken in a separate test tube and the remainder centrifuged on an IEC centra — 7 (International Equipment Company, Needham Heights, MA, USA) for 15 min at 1000*g* to separate plasma and red blood cells. Heparin was used as an anticoagulant. All samples were stored at 4°C until they were analysed.

Extraction

For the extraction of the drug from plasma, whole blood and red blood cells, 100 μl of sulphamethoxazole (IS) (5 μg base per 100 μl), 0.5 ml of distilled water, 100 μl of phosphate buffer (pH 3.40) and 6 ml of ethylene dichloride were added to a 0.5 ml aliquot of the sample (standard or analysis). The tubes were shaken for 20 min on orbital mixer (Denley, Billingshurst, UK) and then centrifuged at 1000*g* for 10 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 200 μl of the mobile phase and 20 μl of this solution was injected for HPLC analysis.

Calibration

Calibration curves were prepared by analysing 0.5 ml samples of plasma, whole blood and red blood cells spiked with known amounts of sulphadoxine. The range of standards was 0.5–100 μg ml^{-1} of the sample. Peak height ratios for sulphadoxine to sulphamethoxazole were used for calibration. Calibration standards were run on each day of analysis.

Recovery and reproducibility

The recovery (extraction yield) was determined at concentrations of 2.5, 10, 25 and 50 μg of sulphadoxine per ml of plasma/whole blood/red 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 compound.

Results and Discussion

Various proportions of acetonitrile, methanol, perchloric acid and water as mobile phase were used to achieve the separation of sulphadoxine, sulphalene, sulphamethoxazole (IS), pyrimethamine and other common antimalarial drugs like chloroquine, primaquine,

quinine, quinidine, amodiaquine and dapsone. It was found that the separation was best achieved using acetonitrile–methanol–(1 M) perchloric acid–water (30:9:0.8:95, v/v/v/v) as mobile phase on μ-Bondapak C_{18} reversed-phase column with a flow of 1.5 ml min^{-1}. The behaviour of sulphadoxine, pyrimethamine, sulphamethoxazole, sulphalene, chloroquine and primaquine with the change in the mobile phase composition of different solvents, acetonitrile, methanol, perchloric acid and water is given in Fig. 1(a), (b), (c) and (d), respectively. An increase in the proportion of acetonitrile decreases the retention, while an increase in the proportion of perchloric acid or water increases the retention of all antimalarials under study following reversed-phase and ion pair mechanism theory. Methanol behaved in a different way, i.e. an increase in the proportion of methanol first decreased the retention and later increased the retention of all antimalarials (Fig. 1b).

The capacity factors (k') of most common antimalarial drugs detected by UV (254 nm) are given in Table 1 which clearly shows that chloroquine, primaquine, amodiaquine, sulphalene, sulphamethoxazole and pyrimethamine do not interfere in the detection of sulphadoxine by this method.

Table 1
Capacity factors of various antimalarials

Antimalarial	k'
Sulphadoxine	2.54
Pyrimethamine	7.70
Sulphamethoxazole	3.03
Sulphalene	1.97
Chloroquine	2.20
Primaquine	3.43
Quinine	1.51
Quinidine	1.17
Amodiaquine	1.96
Dapsone	1.81

Mobile phase, acetonitrile–methanol–(1M) perchloric acid–water (30:9:0.8:95, v/v/v/v); flow rate, 1.5 ml min^{-1}; UV detection wavelength, 254 nm; column μ BondaPak C_{18} (reversed-phase).

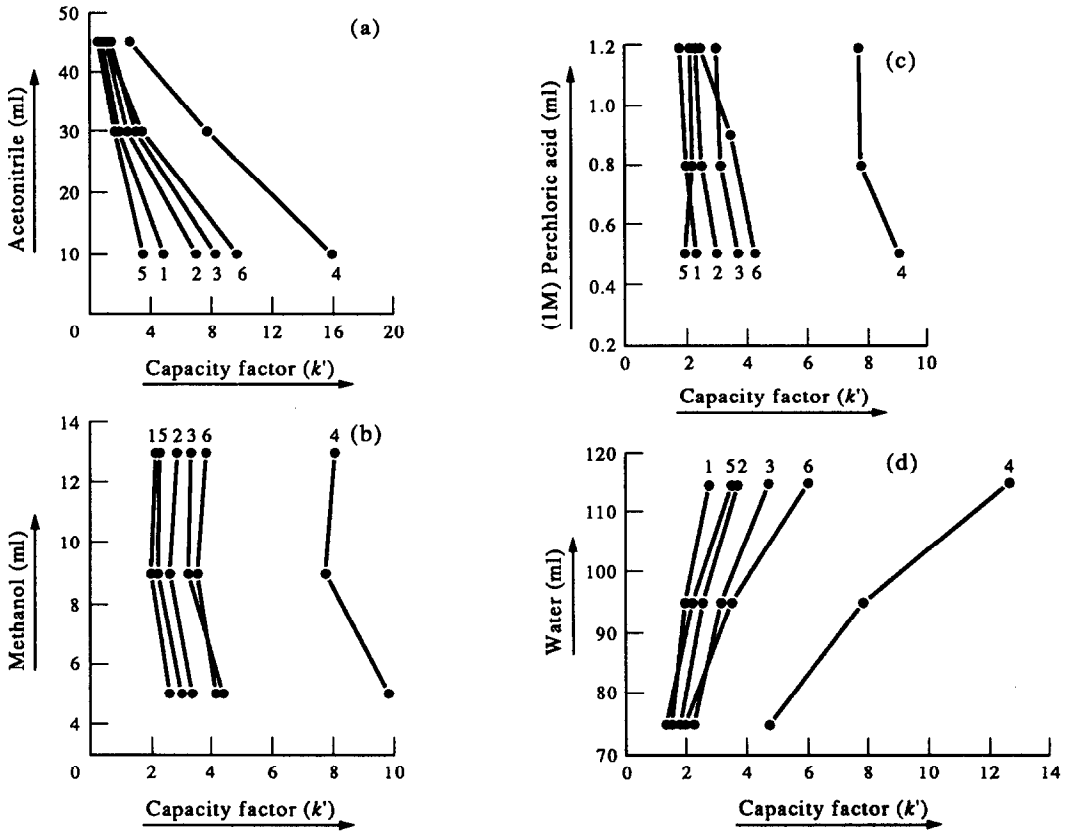

Figure 1
Behaviour of chloroquine(1), sulphalene(2), sulphadoxine(3), sulphamethoxazole(4), primaquine(5) and pyrimethamine(6) with changes of concentration of acetonitrile (a), methanol (b), perchloric acid (c) and water (d) in the mobile phase, acetonitrile–methanol–perchloric acid (1 M)–water (30:9:8:95, v/v/v/v).

During the study, a large number of calibration curves were obtained. All were linear and the correlation coefficients were always above 0.990. The limit of quantification was 50 ng ml^{-1} well above the high therapeutic concentrations of sulphadoxine. Within-day and day-to-day coefficients of variation (CV) averaged 2.1 and 6.45%, respectively. The average extraction recovery from plasma, red blood cells and whole blood was 90.28, 92.05 and 94.69, respectively (Table 2). It was also noted that recovery from aqueous solution was higher when the test tubes were silanized with Aguasil, however, the extraction recoveries from any blood medium were not affected by silanization.

Figure 2 shows the chromatographic behaviour of a blank plasma extract from a healthy volunteer before administration of the drug and plasma extract of a patient on D15 after oral administration of two tablets of Fansidar. Chromatographic behaviour of extracts from red blood cells and whole blood was similar. Some endogenous peaks from plasma, red blood cells and whole blood appeared in the chromatogram but they do not

Table 2
Extraction recovery of HPLC method for sulphadoxine in plasma, whole blood and red blood cells

	Recovery (mean ± SD, n = 4) (%)		
Concentration ($\mu g\ ml^{-1}$)	Plasma	Whole blood (filter paper)	Red blood cells
50.00	90.19 ± 7.05	95.03 ± 1.83	91.93 ± 6.69
25.00	91.14 ± 7.39	97.03 ± 1.65	92.92 ± 2.87
10.00	87.05 ± 3.23	95.90 ± 2.12	93.40 ± 3.12
2.5	92.74 ± 2.89	90.60 ± 1.89	89.98 ± 3.24
Mean ± SD	90.28 ± 2.39	94.69 ± 2.71	92.05 ± 1.51

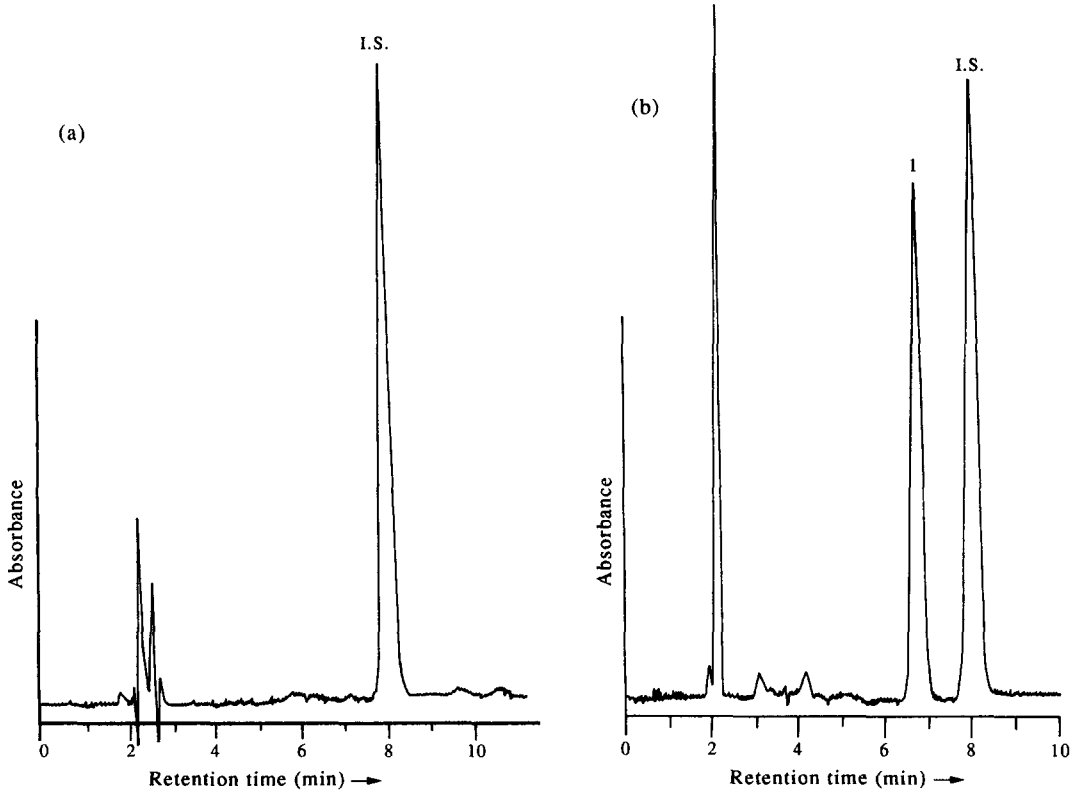

Figure 2
(a) Chromatogram of a blank plasma extract containing sulphamethoxazole (IS) obtained from a volunteer before drug administration. (b) Chromatogram of a plasma extract taken on D15 after oral administration of two tablets of Fansidar. Peaks: 1 = sulphadoxine (26.52 $\mu g\ ml^{-1}$).

interfere in the determination of sulphadoxine. It is worth pointing out that due to low extraction recovery of N^4-acetyl sulphadoxine and pyrimethamine by Edstein method [5] and low metabolism of sulphadoxine to N^4-acetyl sulphadoxine in the body. N^4-acetyl sulphadoxine and pyrimethamine could not be detected by this chromatographic system.

The mean sulphadoxine concentrations in plasma, red blood cells and whole blood of eight healthy and 50 *P. falciparum* malaria cases on D2, D7 and D15 after administration of two tablets of Fansidar as therapeutic dose are given in Tables 3 and 4. The mean sulphadoxine concentration was higher in plasma than whole blood or red blood cells in healthy as well as in *P. falciparum* cases. This is explained by high protein binding of the drug which is acidic and does not accumulate in erythrocytes. The mean sulphadoxine plasma concentration in healthy volunteers was the same as reported by Weidekamm *et al.* [14]. The inter-individual variations of sulphadoxine concentration in different blood components were between 2 and 3-fold which is well in accordance with earlier reports [10, 11, 15].

Statistical comparison of mean sulphadoxine concentrations on D2, D7 and D15 in plasma, red blood cells and whole blood of *P. falciparum* cases and healthy volunteers showed significant difference in the mean values of plasma and whole blood on D2 (t plasma = 4.67; $P < 0.001$; t whole blood = 6.44; $P < 0.001$) and on D7 (t plasma = 3.7; $P < 0.01$; t whole blood = 2.5, $P < 0.05$) while insignificant on D15 (t plasma = 1.63; $P > 0.1$; t whole blood = 0.39, $P > 0.5$). Mean sulphadoxine concentration in red blood cells was found similar in both the cases. Schapira *et al.* [16] have reported the same sulphadoxine concentrations in serum of *P. falciparum* and healthy cases. However, Sarikabhuti *et al.* [10] showed higher sulphadoxine concentration in plasma of *P. falciparum* cases as compared to healthy volunteers. The higher plasma concentration of sulphadoxine in *P. falciparum* cases than in healthy cases may be due to changes in drug disposition in diseased state, resulting in

Table 3
Concentrations of sulphadoxine in plasma, whole blood and red blood cells in healthy volunteers

	Concentration* (mean ± SD) (μg ml^{-1})		
	Day 2	Day 7	Day 15
Plasma	73.87 ± 9.39 (62.53–86.50)	43.91 ± 6.01 (30.71–52.70)	33.93 ± 4.44 (26.29–38.98)
Whole blood	54.28 ± 3.89 (50.10–60.67)	33.24 ± 4.99 (23.09–42.06)	26.97 ± 3.97 (20.82–31.52)
Red blood cells	12.24 ± 2.16 (9.66–15.92)	6.96 ± 1.69 (4.45–9.45)	5.84 ± 1.71 (3.43–8.26)
W.B. plasma	0.73	0.75	0.79
R.B.C. plasma	0.16	0.16	0.17

*Average of eight healthy cases.
†Concentration range in parentheses.

Table 4
Sulphadoxine concentration in plasma, whole blood and red blood cells in *Plasmodium falciparum* patients

	Concentration* (mean ± SD) (μg ml^{-1})		
	Day 2	Day 7	Day 15
Plasma	107.31 ± 15.20 (83.00–144.62)†	75.41 ± 14.24 (43.42–98.67)	39.79 ± 7.72 (25.87–57.26)
Whole blood	67.72 ± 11.82 (50.75–100.41)	44.44 ± 9.73 (30.62–75.07)	24.70 ± 4.46 (10.49–31.13)
Red blood cells	11.73 ± 3.73 (6.30–19.04)	7.00 ± 1.94 (3.57–10.99)	4.41 ± 1.37 (2.24–7.70)
W.B. plasma	0.63	0.59	0.62
R.B.C. plasma	0.10	0.09	0.10

*Average of 50 *P. falciparum* cases.
†Concentration range in parentheses.

an altered protein binding, tissue distribution and increase in the volume of distribution in *P. falciparum* cases [10]. Sulphadoxine concentrations were also higher in plasma than in whole blood in *P. falciparum* as well as healthy cases. This is due to the fact that about 90% of sulphadoxine is bound to albumin, which is found in the plasma fraction and only 4% is distributed in erythrocytes [17]. Furthermore, during the course of infection, profound changes occur in the distribution of plasma protein; albumin, haptoglobin and transferrin levels falls while Gc — globulin, α_1 — anti-chymotrypsin, α_1 — β-glycoprotein and C — reaction protein levels increases [18]. Therefore, sulphadoxine concentration in plasma of malaria cases differ due to its difference in binding with plasma protein. Higher sulphadoxine concentration in malaria cases as compared to healthy volunteers implies that sulphadoxine also binds strongly with other proteins besides albumin. Sulphadoxine concentrations in parasitized erythrocytes in *in vitro* was higher than in non-parasitized erythrocytes [19], however, we did not find significant difference in the concentration of sulphadoxine in red blood cells of *P. falciparum* malaria cases and healthy volunteers.

The mean sulphadoxine concentration ratios in whole blood to plasma in healthy cases on D2, D7 and D15 were 0.73, 0.75 and 0.79 while in *P. falciparum* cases were 0.63, 0.58 and 0.62, respectively. The ratio remained stable from D2 to D15. The lower values in *P. falciparum* cases are due to high sulphadoxine concentration in plasma in *P. falciparum* cases than healthy volunteers. A median whole blood to plasma concentration ratio of 0.57–0.62 has been found using HPLC methods [1, 8, 9]. Recently Hellgren *et al.* [11] have reported a ratio of 0.72 in children with *P. falciparum* malaria in Tanzania. The probable reason for the difference is due to a change in the haematocrit in different subjects [11]. The mean sulphadoxine concentration ratios of red blood cells to plasma on D2, D7 and D15 of *P. falciparum* cases were 0.11, 0.09 and 0.10, while of healthy volunteers they were 0.16, 0.16 and 0.17, respectively. Since the mean red blood cells' sulphadoxine concentrations in *P. falciparum* and healthy cases were similar, therefore, the difference in the ratio in both the cases is mainly due to the difference in the plasma concentrations. Poor distribution of sulphadoxine in erythrocytes as compared to plasma resulted in a very low value of the concentration ratio of red blood cells to plasma.

Conclusion

The chromatographic system described here is sensitive and specific for the determination of sulphadoxine in different blood components. The separation followed reversed-phase phenomenon with ion pair mode. The retention time of all antimalarials under study can be modified by a slight change in methanol proportion in the mobile phase to get the best separation. Our study clearly shows a higher plasma sulphadoxine concentration compared to whole blood or red blood cells. Significant differences in sulphadoxine concentrations in plasma and whole blood were observed between healthy and *P. falciparum* malaria cases. Sulphadoxine accumulates very little in red blood cells.

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