Journal of Chromatography, 563 (1991) 333–340
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5653

Determination of sulfalene in plasma, red blood cells and whole blood by high-performance liquid chromatography

VIRENDRA K. DUA* and REEMA SARIN

Malaria Research Centre (Field Station), BHEL Complex, Ranipur, Hardwar-249403 (India)

and

V. P. SHARMA

Malaria Research Centre, 22-Sham Nath Marg, Delhi-110054 (India)

(First received April 19th, 1990; revised manuscript received September 28th, 1990)

ABSTRACT

A normal phase high-performance liquid chromatographic method using dichloromethane–methanol–perchloric acid (1 *M*) (96:9:1, v/v) at a flow-rate of 1 ml/min on a Nucleosil 100-7 column (250 × 8 × 4 mm) and UV detection at 254 nm, has been developed to determine the concentration of sulfalene in plasma, red blood cells and whole blood after oral administration of the antimalarial drug metakelfin. The coefficient of variation was 7.1% and the extraction recovery was 82%. Mean concentrations of sulfalene on days 1, 7 and 15 were: 49.56, 10.46 and 2.24 μg/ml in plasma, 25.02, 4.34 and 0.84 μg/ml in red blood cells and 21.12, 4.44 and 1.00 μg/ml in whole blood, respectively. Quinine, chloroquine, desethylchloroquine, mefloquine, primaquine, sulfadoxine, pyrimethamine and dapsone did not interfere in the detection of sulfalene.

INTRODUCTION

The emergence of *Plasmodium falciparum* resistance to many antimalarials is becoming a severe problem all over the world. Fansidar [500 mg of sulfadoxine (I) plus 25 mg of pyrimethamine (II)] and Metakelfin [500 mg of sulfalene (III, Fig. 1) plus 25 mg of pyrimethamine] are frequently used for prophylaxis and treatment of malaria in areas where chloroquine resistance is present [1,2]. 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 [3].

Recently, Bergqvist and Churchill [4] reviewed the determination of levels of different antimalarials in biological fluids. Many analytical methods, including spectrophotometry [5], thin-layer chromatography (TLC), gas chromatography (GC) [6] and high-performance liquid chromatography (HPLC) [7,8] have been reported for the determination of sulfadoxine in different body fluids. HPLC is by far the preferred technique for the assay of sulphonamides over the spectrophotometric methods or other chromatographic methods owing to its specificity to-

0378-4347/91/$03.50

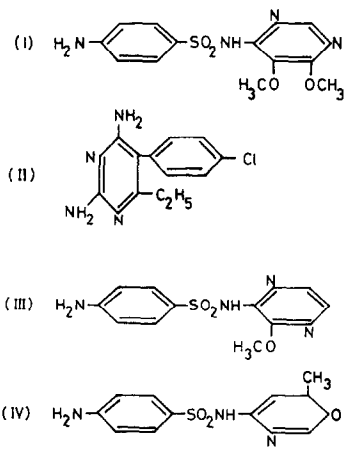

Fig. 1. Molecular structures of sulfadoxine (I), pyrimethamine (II), sulfalene (III) and sulfamethoxazole (IV).

wards the drugs and their metabolites, is speed and its high sensitivity. Many HPLC methods are available for the determination of the level of sulfadoxine in different body fluids [7–10], but no HPLC method is available for the assay of sulfalene (III), which is widely used as Metakelfin for the prophylaxis and treatment of malaria in tropical countries [11].

This paper describes a simple and sensitive method for the determination of the level of sulfalene in plasma, red blood cells and whole blood by normal phase HPLC.

EXPERIMENTAL

Chemicals and standards

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

Pyrimethamine, sulfadoxine, sulfalene and sulfamethoxazole were supplied by ICI (Australia). Stock standard solutions of pyrimethamine (0.25 mg/ml), sulfadoxine, sulfalene and sulfamethoxazole (5 mg/ml each) were prepared separately in methanol. Intermediate and working standard solutions covering the concentration range reported by Edstein [8] were prepared by diluting the stock standard solution with methanol. Sulfamethoxazole was used as the internal standard (I.S.). Solutions were stored at 4°C.

A 12 m*M* 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, U.S.A.) consisting of a 510 pump, a 490 programmable multiwavelength UV detector operated at 254 nm, a WISP 712 automatic sample injector and a Digital 380 data station with Digital printer, was used for analysis. The mobile phase was dichloromethane–methanol–perchloric acid (1 *M*) (96:9:1, v/v), and was pumped at a flow-rate of 1 ml/min at ambient temperature on the Nucleosil 100-7 column (Macherey-Nagel, Düren, F.R.G.), particle size 7 μm (250 × 8 × 4 mm) for the successful separation of pyrimethamine, sulfadoxine, sulfalene and sulfamethoxazole (internal standard, I.S.). The mobile phase was filtered and degassed by ultrasonication (Decon FS100, Hove, U.K.) before use.

Subjects

The subjects in this study were eight healthy volunteers (age range 20–25 years). Two tablets of Metakelfin (Dominion Chemical Industries, Calcutta, India), consisting of 1000 mg of sulfalene and 50 mg of pyrimethamine, were given to each volunteer with no other drug intake history. Intravenous blood (2.5 ml) was drawn from each person on days 0 (D0), 1 (D1), 7 (D7), 15 (D15) and 21 (D21). EDTA was used as an anticoagulant. Of the 2.5 ml of blood drawn, 0.5 ml of whole blood was taken in a separate test-tube and the remainder was centrifuged on IEC centra-7 (Internal Equipment Company, Needham Heights, MA, U.S.A.) for 15 min at 1000 *g* to separate plasma and red blood cells. All samples were stored at 4°C.

Extraction

For the extraction of the drugs from plasma, red blood cells and whole blood, the procedure of Edstein [8] was followed. Briefly, to an aliquot of 0.5 ml of the 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, U.K.) and 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, U.S.A.). The residue was dissolved in 100 μl of the mobile phase, and 20 μl of this solution were injected for HPLC analysis.

Calibration

Calibration curves were prepared by analysing 0.5-ml plasma/red blood cells/whole blood samples spiked with known amounts of the compound. The range of standards was 0.5–100 μg/ml of the sample. Peak-area ratios of sulfalene to sulfamethoxazole were used for calibration.

Recovery and reproducibility

The recovery (extraction yield) was determined at concentrations of 2.5, 5.0,

20, 30 and 40 μg of sulfalene per ml of plasma by comparing peak areas with areas obtained by direct injection of pure standards. Within-day reproducibility was evaluated by analysing plasma standards containing 5, 10, 20, 30 and 40 μg of sulfalene per ml on four occasions.

RESULTS AND DISCUSSION

The behaviour of pyrimethamine, sulfadoxine, sulfalene and sulfamethoxazole with change in the mobile phase proportion of dichloromethane, methanol and perchloric acid is given in Fig. 2A, B and C, respectively. An increase in the

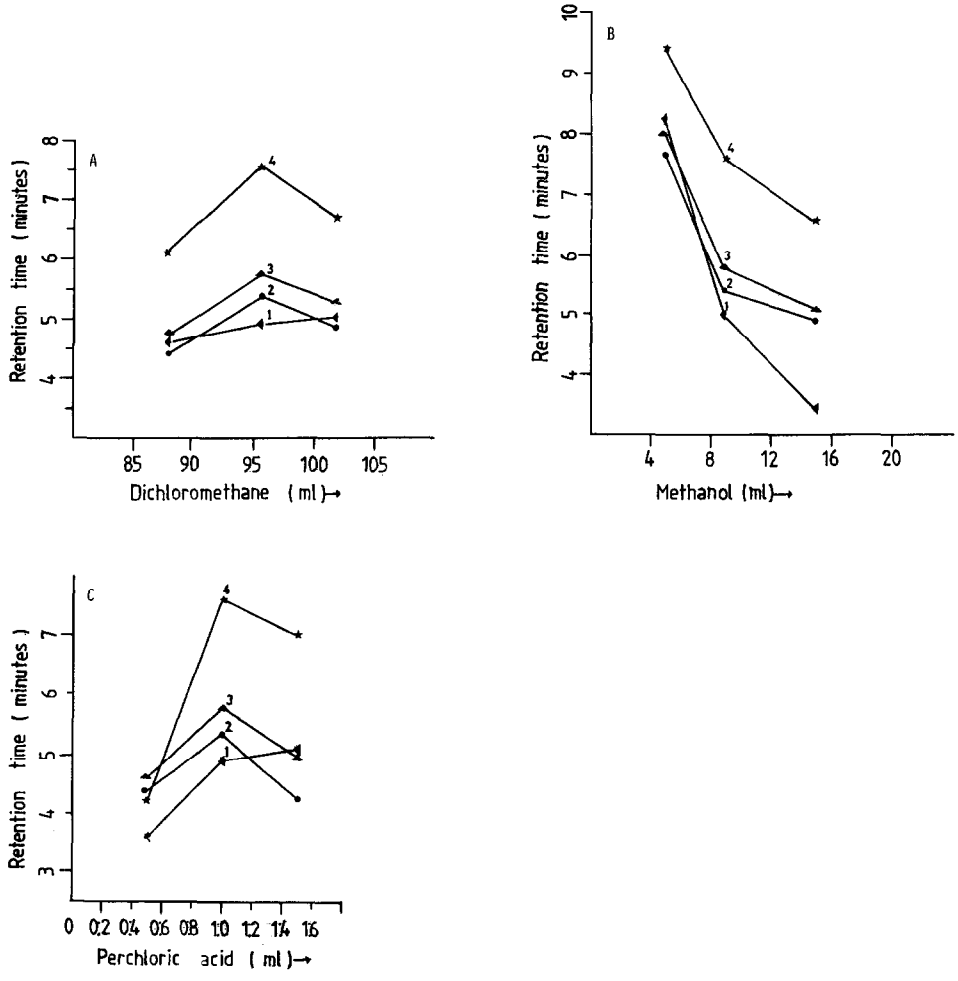

Fig. 2. Behaviour of pyrimethamine (1), sulfadoxine (2), sulfalene (3) and sulfamethoxazole (4) with changes of concentration of dichloromethane (A), methanol (B) and perchloric acid (C) in the mobile phase, dichloromethane–methanol–perchloric acid (1 *M*).

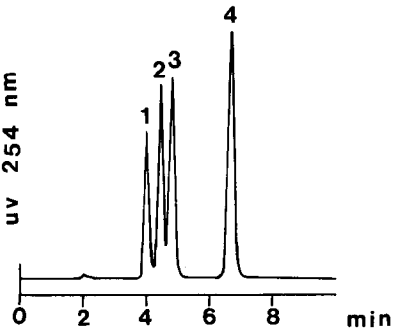

Fig. 3. HPLC separation of pyrimethamine (1), sulfadoxine (2), sulfalene (3) and sulfamethoxazole (4); 0.2 μg of each drug was injected.

dichloromethane or perchloric acid proportion increased the retention times of most of the antimalarials under study, whereas methanol had the opposite effect. It was found that dichloromethane–methanol–perchloric acid (1 *M*) in the proportion of 96:9:1 gave the best separation of pyrimethamine, sulfadoxine, sulfalene and sulfamethoxazole.

HPLC separation of these antimalarials on Nucleosil 100-7 column is shown in Fig. 3. The relative retention times (k') of most common antimalarial drugs are given in Table I, which clearly shows that chloroquine, desethylchloroquine, primaquine, quinine, mefloquine and dapsone do not interfere in the separation of pyrimethamine, sulfadoxine, sulfalene and sulfamethoxazole. The relative retention times of pyrimethamine, sulfadoxine and sulfalene are close but the difference between the k' values of pyrimethamine and sulfalene is sufficient for the

TABLE I

RELATIVE RETENTION TIMES OF DIFFERENT ANTIMALARIALS

Mobile phase, dichloromethane–metahnol–perchloric acid (1 *M*) (96:9:1); flow-rate, 1 ml/min; UV detection, 254 nm; column, Nucleosil 100-7 (normal phase).

Antimalarial	k'
Sulfalene	2.55
Pyrimethamine	2.24
Sulfadoxine	2.40
Sulfamethoxazole	3.38
Quinine	3.13
Chloroquine	3.11
Primaquine	4.87
Dapsone	5.11
Mefloquine	1.64
Desethylchloroquine	3.62

TABLE II

PRECISION OF THE HPLC METHOD FOR SULFALENE IN PLASMA (SPIKED SAMPLES)

	Concentration (μg/ml)	*n*	C.V. (%)
Within-day	20.0	5	2.4
	40.0	5	3.8
Mean ± S.D.	3.1 ± 0.98		
Day-to-day	5.0	4	14.2
	10.0	4	7.7
	20.0	4	2.3
	30.0	4	5.2
	40.0	4	6.2
Mean ± S.D.	7.1 ± 4.4		

determination of sulfalene in plasma, red blood cells and whole blood by this method. However, the separation of pyrimethamine and sulfadoxine can be improved by using dichlormethane–methanol–perchloric acid (1 *M*) in the proportion of 96:9:0.5

Within-day and day-to-day coefficients of variation (C.V.) averaged 3.1 and 7.1% respectively (Table II). The extraction recovery was 82% for sulfalene (Table III). Fig. 4 shows the chromatographic behaviour of a blank plasma extract and a plasma extract taken on the seventh day from a volunteer after oral administration of two tablets of Metakelfin. Some endogenous compound peaks from plasma, red blood cells and whole blood appeared in the chromatogram, but they appear well before pyrimethamine, sulfadoxine, sulfalene and sulfamethoxazole

TABLE III

RECOVERY OF THE HPLC METHOD FOR SULFALENE IN PLASMA

Concentration (μg/ml)	Recovery (mean ± S.D., $n = 4$) (%)
2.5	67 ± 5.9
5.0	78 ± 4.7
20.0	84 ± 2.1
30.0	86 ± 3.2
40.0	95 ± 1.8
Mean ± S.D.	82 ± 10.36

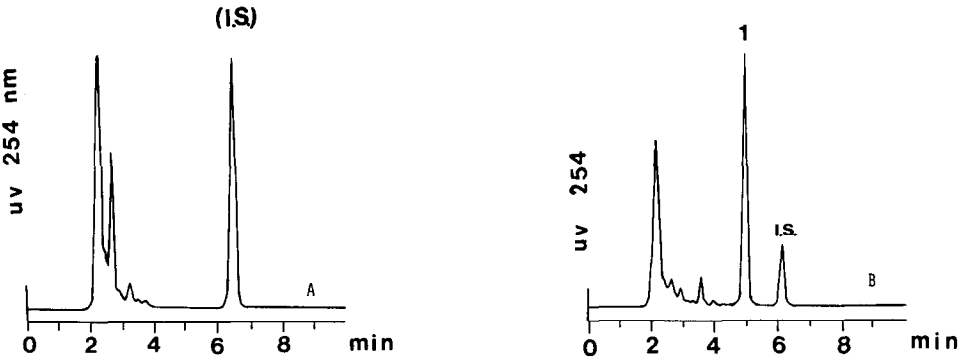

Fig. 4. (A) Chromatogram of a blank plasma extract containing sulfamethoxazole (I.S.). (B) Chromatogram of a plasma extract taken on the seventh day after oral administration of two tablets of Metakelfin. Peaks: 1 = sulfalene; I.S. = sulfamethoxazole.

and thus do not interfere in the determination of sulfalene. The possibility of overlapping of the sulfalene peak with those of metabolites was ruled out by isolating preparatively the peak corresponding to sulfalene from the volunteer's plasma sample and rechromatography of this peak on a reversed-phase C_{18} column, which shows a single peak (manuscript in preparation).

The average concentration profile of sulfalene in plasma, red blood cells and whole blood of eight volunteers after administration of two tablets of metakelfin is given in Table IV. Mean concentrations of sulfalene on D1, D7, and D15 are 49.56, 10.46, and 2.24 μg/ml in plasma, 25.02, 4.34 and 0.84 μg/ml in red blood cells and 21.12, 4.44 and 1.00 μg/ml in whole blood, respectively. Sulfalene was not detected on D21 in any of the blood media. It may be noted that the average plasma and whole blood concentrations of sulfadoxine after D7 were 39.6 and

TABLE IV

SULFALENE CONCENTRATIONS IN PLASMA, RED BLOOD CELLS AND WHOLE BLOOD

Sample	Concentration (mean ± S.D., n = 8) (μg/ml)		
	Day 1	Day 7	Day 15
Plasma	49.56 ± 6.42	10.46 ± 2.16	2.24 ± 0.50
RBC[a]	25.02 ± 4.16	4.34 ± 1.62	0.84 ± 0.30
Whole blood	21.12 ± 7.02	4.44 ± 1.28	1.00 ± 0.36
RBC/plasma	0.5048	0.4149	0.375
WB/plasma	0.4261	0.4248	0.4464

[a] Red blood cells.

23.76 μg/ml, whereas the concentration of sulfalene on D7 in our case was 10.46 μg/ml and 4.44 μg/ml in plasma and whole blood, respectively. The ratio of the sulfalene concentrations in whole blood and plasma on D1, D7 and D15 was 0.42, 0.42 and 0.44, respectively. Bergqvist *et al.* [10] and Edstein [8] have found the ratio of whole blood to plasma concentrations to be 0.57–0.62 for sulfadoxine. The lower values of plasma and whole blood concentrations and the whole blood to plasma ratio for sulfalene in our studies may be due to the fact that sulfalene has less protein binding (68%) and a shorter half-life (65 h) than sulfadoxine, which has a protein binding of 90% and a half-life of 135 h [12].

The average ratio of the concentrations of sulfalene in red blood cells and plasma on D1, D7 and D15 were 0.50, 0.41, and 0.37, respectively. Trenholme *et al.* [13] have reported the sulfalene concentration ratio in erythrocytes to concomitant (total concentration including metabolites) in plasma as 32–41% after 8–120 h, respectively.

Berneis and Boguth [14] studied the distribution of sulfonamides between the red blood cells and plasma by adding the drug directly to the blood, and found the ratio to be 0.3–0.4 using spectrophotometry. The higher value of the ratio in our case is certainly due to the selectivity of HPLC method for the drug and its metabolites.

REFERENCES

1 E. B. Doberstyn, P. Phintuyothin, S. Noeypatimanondh and C. Teerakiartkamjorn, *Bull. WHO*, 57 (1979) 275.
2 T. Chongsuphajaisiddhi, *Southeast Asian J. Trop Med. Public Health*, 11 (1980) 524.
3 U. Hellgreen, Ph. D. Thesis, Karolinska Institute, Stockholm, 1990.
4 Y. Bergqvist and F. C. Churchill, *J. Chromatogr.*, 434 (1988) 1.
5 A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, 128 (1939) 537.
6 M. Bonini, F. Mokofio and S. Barazi, *J. Chromatogr.*, 224 (1981) 332.
7 C. Midskov, *J. Chromatogr.*, 308 (1984) 217.
8 M. Edstein, *J. Chromatogr.*, 305 (1984) 502.
9 Y. Bergqvist and M. Eriksson, *Trans. R. Soc. Trop. Med. Hyg.*, 79 (1985) 297.
10 Y. Bergqvist, E. Hjelm and L. Rombo, *Ther. Drug Monit.*, 9 (1987) 203.
11 S. Das, B. N. Barkakaty, J. R. Roy, A. K. Guha, K. C. Rastogi, S. R. Dwivedi, R. G. Roy and S. Pattanayak, *Indian J. Malariol.*, 18 (1981) 109.
12 H. J. Scholer, R. Leimer and R. Richle, in W. Peters and W. H. G. Richards (Editors), *Antimalarial Drugs*, Vol. II, Springer, Berlin, 1984 p. 123.
13 G. N. Trenholme, R. F. Williams, H. Fischer, P. E. Carson, K. H. Rickmann, *Ann. Intern. Med.*, 82 (1975) 219.
14 K. Berneis and W. Boguth, *Chemotherapy*, 22 (1976) 390.