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Short Communication

Simultaneous determination of dapsone, monoacetyldapsone and pyrimethamine in whole blood and plasma by high-performance liquid chromatography

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ABSTRACT

A sensitive, selective and rapid reversed-phase high-performance liquid chromatographic method was developed for the simultaneous analysis of dapsone, monoacetyldapsone and pyrimethamine in human whole blood and plasma. The procedure involved extraction of the compounds and the internal standard, monopropionyldapsone, with *tert.*-butylmethyl ether under alkaline conditions. A newly marketed column, Supelcosil LC-ABZ (Supelco, 15 cm × 4.6 mm I.D.), was employed. The mobile phase, consisting of acetonitrile–methanol–phosphate buffer (2:1:7, v/v/v), was delivered at a flow-rate of 1.2 ml/min, and ultraviolet absorbance was monitored at 286 nm. The limit of determination using a 150-μl sample was 10 ng/ml (40 n*M*) for dapsone and pyrimethamine and 8 ng/ml (28 n*M*) for monoacetyldapsone. Given that only a small amount of blood is required in this method, it could now be applied in studies involving blood level monitoring and pharmacokinetics in children on Maloprim (dapsone–pyrimethamine) prophylaxis in malaria endemic areas.

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INTRODUCTION

Maloprim is a combination of the drugs dapsone (DDS) and pyrimethamine (PYR), which have synergistic antimalarial effect. It inhibits the folic acid metabolism of malarial parasites. Maloprim is effective against chloroquine-resistant *Plasmodium falciparum* malaria. It has been used successfully for long-term malaria prophylaxis in African children [1,2]. However, no pharmacokinetic information was given in these previous studies. Studies that provide pharmacokinetic data have been conducted in healthy adults [3–5] using HPLC for the simultaneous analysis of Maloprim in plasma and serum samples. The only study that provides pharmacokinetic data for Maloprim in plasma, serum, whole blood and red blood cells [5] utilized sample volumes of 1 ml of the first two and 0.5 ml of the last two; the analysis employed two different chromatographic conditions. The method was sensitive enough to estimate trough concentrations of the drug, but the amount of sample and the need for repeated venepuncture would not be popular in children.

The aim of our study was, therefore, to find a sensitive HPLC method that can be used for the simultaneous analysis of DDS and PYR in small amounts of whole blood and plasma. Such a method could be further developed for field application in studies involving children taking Maloprim for malaria prophylaxis. Monitoring of Maloprim blood levels could determine whether breakthroughs are due to inadequate drug concentrations or resistant parasites. Potentially toxic levels might also be disclosed.

EXPERIMENTAL

Instrumentation and chromatographic conditions

A Hewlett-Packard Model 1084B liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a variable-wavelength UV detector was used. The wavelength was set at 286 nm and sensitivity at 0.012 a.u.f.s. Injection was made with an HP autosampler. The column was a 15 cm × 4.6 mm I.D., 5 μm particle size, Supelcosil LC-ABZ column (Supelco, Bellefonte, Philadelphia, PA, USA). The mobile phase consisted of acetonitrile–methanol (2:1, v/v) in phosphate buffer (25 m*M*) that had been adjusted to pH 2.3 with orthophosphoric acid. Mobile phase was filtered through a 0.45-μm Millipore filter before use. The mobile phase was delivered at a flow-rate of 1.2 ml/min. The chromatographic analysis was run at ambient temperature. The mobile phase was recycled for a maximum of one week or 100 injections. Alternatively, when drifts in the retention times were above 2.8 min for PYR and 9.5 min for monopropionyldapsone (MPD) the mobile phase was changed.

Chemicals and reagents

Acetonitrile, methanol, ethanol and *tert.*-butylmethyl ether were of HPLC grade (LiChrosolv, Merck, Darmstadt, Germany). Orthophosphoric acid was of analytical grade (Merck). Sodium hydroxide and sterile water were from Rigshospitalet (Copenhagen, Denmark). The reference compounds were: DDS (Sigma, St Louis, MO, USA), PYR (Roche, Basle, Switzerland), monoacetyldapsone (MADDS) and MPD, which were synthesized in the laboratory. MADDS was prepared by refluxing 2 mmol each of DDS and acetic anhydride in the presence of ethyl acetate for 10 min. The internal standard, MPD, was synthesized by reacting 2 mmol of DDS with 2 μmol of propionic anhydride for 10 min under reflux with ethyl acetate. In both reaction processes DDS and ethyl acetate were heated together and the appropriate anhydride was added just when the mixture was beginning to boil. MADDS and MPD were separated from excess DDS and other products by preparative TLC (Silica gel 60 F-254 20 cm × 20 cm × 2 mm precoated TLC plates, Merck) using ethyl acetate as the developing solvent for MADDS and a mixture of equal volumes of chloroform, *n*-heptane and ethanol for MPD. Structures were confirmed by MS while purity was checked by HPLC. Stock solutions of the different compounds were prepared in ethanol. The concentrations were 5 mg/ml (20 m*M*) DDS, 0.5 mg/ml (2 m*M*) PYR and 2 mg/ml (6.9 m*M*) MADDS. A working solution containing 0.2 mg/ml DDS,

0.04 mg/ml MADDS and 0.02 mg/ml PYR was prepared in water. The stock solutions were stored at −20°C, while aqueous working solutions were kept at 4°C.

Sample extraction

To a 150-μl whole-blood or plasma sample were added an equal volume of water and 25 μl of the internal standard (MPD) which had been diluted twenty times in ethanol. The contents were mixed on an electronic shaker (Type VX 2E, Janke & Kunkel, Staufen, Breisgau, Germany) for 10 min at a speed of 2200 vibrations per min. A 100-μl volume of sodium hydroxide (2 *M*) and 3 ml of *tert.*-butylmethyl ether were then added and extraction was carried out on a mechanical shaker for 15 min. After centrifugation at 1200 *g* for 10 min, the clear organic layer was transferred to a clean tube using a Pasteur pipette. Evaporation to dryness was by a stream of air at 37°C. The residue was dissolved in 100 μl of mobile phase. After vortex-mixing for 20 s and centrifugation (1200 *g*, 2 min), an 80-μl aliquot was injected into the chromatograph.

Calibration procedure

Standard curves were prepared by duplicate analysis of 150-μl whole-blood or plasma samples spiked with different concentrations of the compounds. The concentrations ranged from 200 to 6400 ng/ml for DDS, 40 to 1240 ng/ml for MADDS and 20 to 640 ng/ml for PYR. Peak-area ratios of DDS and MADDS to the internal standard and peak-height ratios of PYR to the internal standard were plotted against concentrations to check linearity range. A reproducible linear relationship was observed with PYR peak heights instead of peak areas. Two calibrators were always included on each analysis.

Method precision and accuracy

The precision of the method based on within-day repeatability was determined by replicate analysis of six samples spiked with three different concentrations of DDS, MADDS and PYR. Two calibrators were also run concurrently in duplicate, and the replicate analysis was repeated on three different occasions. The reproducibility (day-to-day variation) of the method was established using the same concentration range as above, but only a single determination of each concentration was made on six different days. The two calibrators were run in duplicate on each day. Accuracy was determined by replicate analysis of two different levels and comparing the difference between spiked value and that actually found.

Recovery

The analytical recovery of DDS, MADDS and PYR was determined by comparing concentrations found after extraction of spiked whole blood or plasma with those obtained by direct injection of the compounds into the mobile phase. The concentration range covered in the different levels was 400–3200, 80–640 and 40–320 ng/ml for DDS, MADDS and PYR, respectively. For the direct injections, the internal standard (25 μl) was evaporated to dryness and then 100 μl (100%) of mobile phase containing the different compounds at the three levels were added. For the extracted samples of the different levels aliquots of exactly 2 ml of the organic layer were evaporated to dryness. Six replicates were run for the levels and 100% of the levels in mobile phase.

Interferences

The selectivity of the method was verified by checking for interference by some antimalarials and some commonly used drugs after subjecting them to the extraction procedure. No interference was seen with either chloroquine or quinine, but proguanil co-elutes with DDS and would thus interfere. Similarly, sulphamethoxazole, trimethoprim and paracetamol showed no interference with the analysis.

RESULTS

Chromatography

Chromatograms of the separation of PYR, DDS, MADDS and the internal standard (MPD) in extracted whole blood and plasma are shown

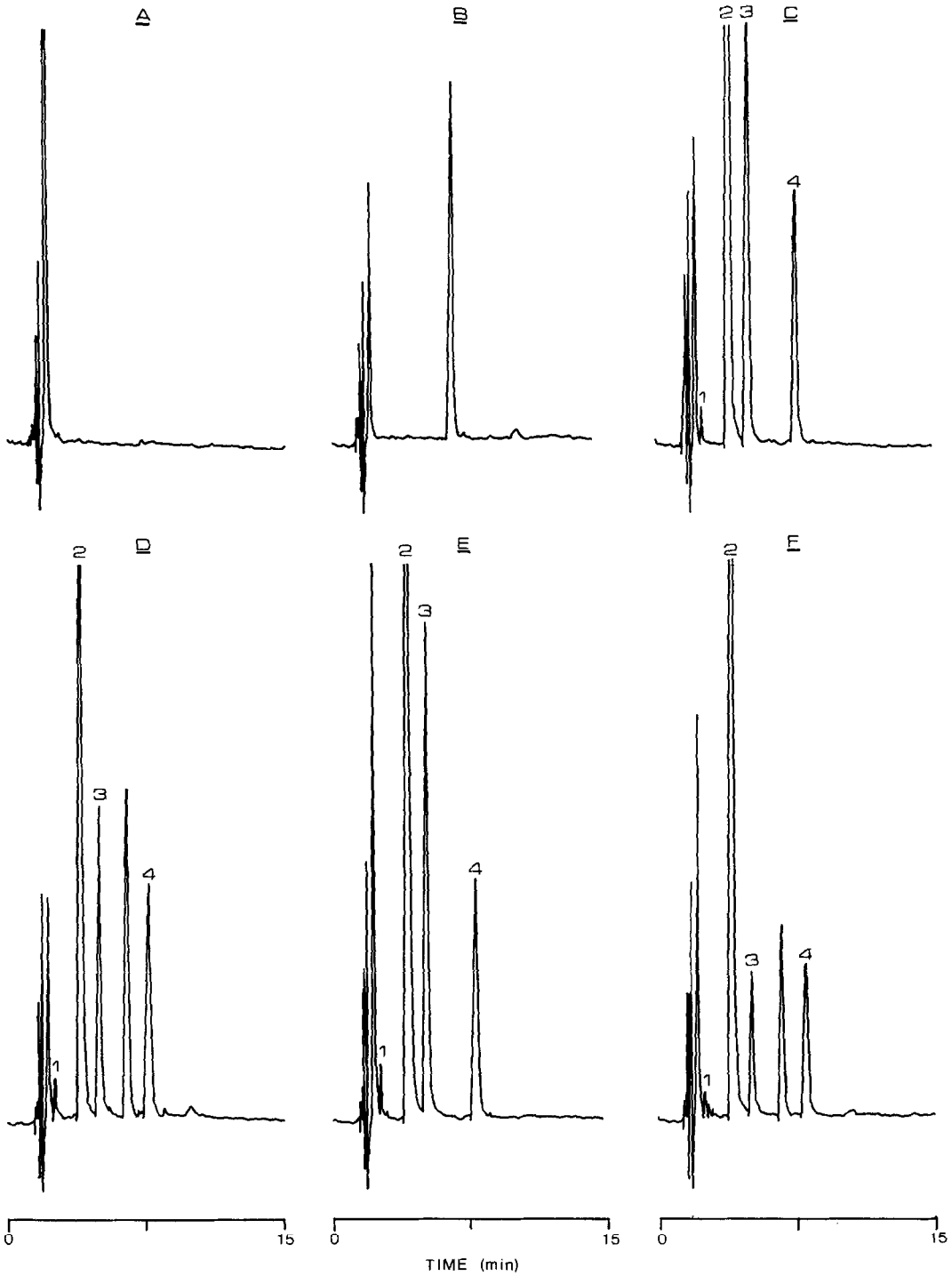

Fig. 1. Representative chromatograms obtained after extraction of (A) blank plasma, (B) blank whole blood, (C) plasma spiked with 80 ng/ml PYR (1), 800 ng/ml DDS (2) and 160 ng/ml MADDS (3). Internal standard, MPD, is peak 4. (D) Whole blood spiked as in C. (E) Plasma sample obtained 4 h after administration of 100 mg of DDS plus 12.5 mg of PYR to a healthy volunteer (concentrations found were: PYR, 108 ng/ml; DDS, 1722 ng/ml; and MADDS, 199 ng/ml). (F) Whole-blood sample taken from the same volunteer as in E (PYR, 86 ng/ml; DDS, 1730 ng/ml; and MADDS, 111 ng/ml).

TABLE I

PRECISION OF THE METHOD FOR DETERMINING DAPSONE, MONOACETYLDAPSONE AND PYRIMETHAMINE IN WHOLE BLOOD AND PLASMA GIVEN AS RELATIVE STANDARD DEVIATION

The number of experiments is given in parentheses. Conversion from ng/ml to nmol/l is obtained by multiplying by the factor 4 for PYR (MW 249) and DDS (MW 248) and 3.5 for MADDS (MW 290).

Matrix	Relative standard deviation (%)								
	DDS			MADDS			PYR		
	400 ng/ml	800 ng/ml	3200 ng/ml	80 ng/ml	160 ng/ml	640 ng/ml	40 ng/ml	80 ng/ml	320 ng/ml
Within-day variation									
Whole blood	4.9 (5)	6.1 (6)	1.8 (4)	6.1 (5)	4.5 (6)	2.3 (4)	2.0 (5)	6.0 (6)	1.3 (4)
Plasma	6.1 (6)	3.2 (6)	1.1 (5)	5.6 (6)	4.3 (6)	3.0 (5)	5.4 (6)	0.8 (6)	1.7 (5)
Day-to-day variation									
Whole blood	2.3 (6)	2.6 (7)	1.4 (5)	5.3 (6)	1.4 (7)	1.2 (5)	2.7 (6)	9.2 (7)	4.0 (5)
Plasma	2.7 (6)	3.5 (6)	1.5 (6)	5.0 (6)	4.7 (6)	5.1 (6)	5.6 (6)	3.9 (6)	1.6 (6)

in Fig. 1A and B. Retention times of 2.5, 3.9, 4.9 and 7.5 min were observed for PYR, DDS, MADDS and MPD, respectively. Endogenous peaks from extracted drug-free whole blood and plasma did not interfere with the drug analysis (Fig. 1C and 1D). Chromatograms of whole blood and plasma samples from a volunteer on a single dose of DDS plus PYR (8:1) are given in Fig. 1E and F. The limit of determination, using replicate analysis of six samples (intra-assay), was 10 ng/ml for DDS and PYR and 8 ng/ml for MADDS (R.S.D. $\leqslant 10\%$) in both whole blood and plasma.

Standard curves, precision, recovery and accuracy

Good linearity was seen, with correlation coefficients $\geqslant 0.997$ for the three compounds in both whole blood and plasma. The intra-assay (within-day) and inter-assay (day-to-day) variation for DDS, MADDS and PYR at three different con-

TABLE II

ANALYTICAL RECOVERY OF THE DRUGS IN WHOLE BLOOD AND PLASMA

DDS			MADDS			PYR		
Concentration (ng/ml)	Recovery (%)	R.S.D.[a] (%)	Concentration (ng/ml)	Recovery (%)	R.S.D. (%)	Concentration (ng/ml)	Recovery (%)	R.S.D. (%)
Whole blood (n = 6)								
400	87	2.1	80	66	3.3	40	113	5.8
800	84	7.2	160	70	5.6	80	93	10.0
3200	72	3.3	640	74	3.6	320	101	3.4
Plasma (n = 6)								
400	92	1.1	80	92	5.9	40	107	5.7
800	92	5.2	160	95	3.1	80	100	4.0
3200	74	5.0	640	73	5.7	320	76	4.5

[a] Relative standard deviation.

TABLE III

ACCURACY OF THE METHOD FOR WHOLE-BLOOD AND PLASMA SAMPLES SPIKED WITH DDS, MADDS AND PYR GIVEN AS PERCENTAGE DEVIATION FROM SPIKED VALUE

Compound	Concentration (ng/ml)	Percentage deviation	R.S.D. (%)
Whole blood (n = 10)			
DDS	800	+1.0	4.7
	3200	+1.5	3.5
MADDS	160	−2.6	4.2
	640	−0.2	3.2
PYR	80	+2.3	4.4
	320	−3.6	3.1
Plasma (n = 10)			
DDS	800	−0.9	3.9
	3200	−2.6	2.5
MADDS	160	+0.1	3.2
	640	−3.4	2.4
PYR	80	−2.1	4.6
	320	−3.5	2.1

centrations are given in Table I. Table II shows the recovery found with the three compounds at two different concentrations; the internal standard recovery was between 85 and 108%. The accuracy of the method for DDS, MADDS and PYR is presented in Table III.

DISCUSSION

The HPLC method just developed for the simultaneous analysis of DDS, MADDS and PYR is quite sensitive. Previously published methods for the simultaneous estimation of these compounds [3,6] require larger sample volumes. The sensitivity observed with our method is similar to that described elsewhere [5,6]. Trough concentrations reported previously [5] were 7.5 ng/ml DDS and 41.5 ng/ml PYR in plasma from healthy volunteers on weekly Maloprim. The same study [5] showed that maximum plasma concentrations of DDS and PYR were 1134 and 116 ng/ml, respectively. Figures given elsewhere [7] for the therapeutic range of DDS in different clinical use are 0.5–5 mg/l. The PYR plasma concentration that can cause inhibition of blood schizogony in sensitive *Plasmodium falciparum* ranges from 10 to 100 ng/ml [8]. This implies our method could be employed in pharmacokinetic studies and blood level estimations in prophylactic studies. Two of the methods described previously [3,6] would probably not be suitable for the analysis of DDS, MADDS and PYR in whole blood unless some modifications are made. Edstein *et al.* [5] employed two separate chromatographic conditions for the estimation of the three compounds in whole blood, red blood cells, plasma and serum. This approach could be unpopular in studies involving children, since repeated blood sampling by venepuncture is required. Considering the small sample volume needed in our method, it seems likely that the method can be optimized for field use, where fingerprick blood sampling would be appropriate.

Diacetyldapsone (DADDS), the synthesis of which is easy, could not be used here as an internal standard as recommended in an earlier study [5] because it co-elutes with an endogenous compound found in whole blood. The use of MPD as an internal standard has been described previously [9,10].

Dichloromethane extraction failed to remove some endogenous substances in whole blood that co-eluted with DDS and MPD. However, *tert.*-butylmethyl ether extracts were clean at the positions where the two compounds eluted. The extraction procedure employed in our method was adopted primarily for steps involving extraction of filter paper-absorbed whole-blood samples. Reliable results for samples not absorbed on filter paper could also be obtained by replacing the 10-min initial mixing step with a few seconds' vortex-mixing.

It can be concluded that the HPLC method described here could provide information on blood levels in cases of drug resistance or breakthroughs during malaria prophylaxis with Maloprim. Although venous blood samples were used in our study, capillary blood samples could possibly be employed.

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