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Note

High-performance liquid chromatographic method for the determination of pyrimethamine and its 3-N-oxide metabolite in biological fluids

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Pyrimethamine—sulphonamide combination therapy is a first choice in areas of chloroquine resistant *Plasmodium falciparum* malaria. The pharmacokinetics of pyrimethamine are poorly understood due to the lack of selectivity of previous analytical methods [1—3].

This paper describes a selective and sensitive microanalytical method for the determination of pyrimethamine in plasma and urine. It is also suitable for the simultaneous determination of pyrimethamine 3-N-oxide (Fig. 1), which has recently been identified as a metabolite of pyrimethamine in the rat [4].

MATERIALS AND METHODS

Chemicals

Pyrimethamine base, 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine, and pyrimethamine 3-N-oxide were supplied by Wellcome U.K. (Beckenham, U.K.).

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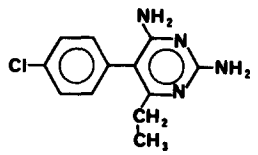

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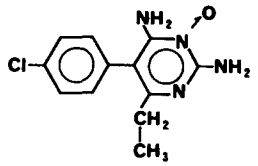

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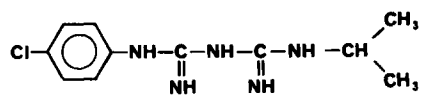

III

Fig. 1. Structural formulae of pyrimethamine (I), pyrimethamine 3-N-oxide (II) and internal standard (III).

Proguanil hydrochloride (Fig. 1), the internal standard, was supplied by ICI Pharmaceuticals (Alderley Edge, U.K.). Ammonia solution (0.88 specific gravity) and orthophosphoric acid were obtained from British Drug Houses (Poole, U.K.). Octanesulphonic acid was supplied by Aldrich (Gillingham, U.K.). All other reagents and solvents were HPLC grade (Fisons, Loughborough, U.K.).

Chromatography

The method was developed on a Spectra-Physics liquid chromatograph. The system consisted of an SP 8770 solvent delivery system, an SP 8750 organiser module equipped with a Rheodyne injection system, and an SP 8300 fixed-wavelength UV absorbance detector fitted with a 254-nm source. The separation was carried out on a Partisil ODS reversed-phase column, 10 μm particle size, from HPLC Technology (Wilmslow, U.K.). The mobile phase consisted of water—acetonitrile—methanol (55:35:10, v/v) containing octanesulphonic acid (0.013 *M*) as an ion-pair reagent, buffered to pH 3.5 with orthophosphoric acid, and flowing at 1.8 ml/min.

Sample treatment procedure

To samples of plasma or urine (10—45 μl) contained in 1.5-ml capacity microcap tubes (L.I.P. Equipment and Services, West Yorkshire, U.K.) were added a methanolic solution of the internal standard (20 μg/ml; 0—20 μl added) and ammonia solution (500 μl). The mixture was extracted twice with ethyl acetate (800 μl) by vortex mixing.

After separation, the combined organic phases were evaporated to dryness under nitrogen at 45°C and reconstituted in methanol (20 μl). A 5—18 μl aliquot was injected onto the column.

Calibration curves, analytical precision and recovery

Standard curves in the range 0.5—20 μg/ml were prepared by adding known quantities of pyrimethamine and pyrimethamine 3-N-oxide to drug-free plasma or urine containing the internal standard (20 μg/ml). Samples were analysed as described above, and the peak height ratio of compound to internal standard was plotted against the corresponding weight ratio. Peak height ratios of experimental samples were also determined and the concentrations calculated from the standard curves.

Recovery of pyrimethamine, its 3-N-oxide, and the internal standard were estimated by comparing the peak height obtained from an extracted plasma sample with that from a methanolic solution containing the same amount of each compound. The intra- and inter-assay precision data were determined for both pyrimethamine and its 3-N-oxide by replicate assays of the same sample.

Calculations

Coefficients of variation (C.V.) for calculation of assay precision were calculated from the ratio of the standard deviation to the mean. Plasma elimination half-life was calculated by regression analysis of the post-distributive log linear portion of the plasma concentration versus time curve. Data are presented as mean ± S.D.

Animal studies

Two groups, A and B, of five mice (male TFW, mean weight 25 g) were provided with a Dixons 41B diet and drinking water ad libitum. Mice of both groups were dosed with pyrimethamine base (75 mg/kg intraperitoneally) which was suspended in 'Tween 80' (5%, v/v). Blood samples were obtained from mice in group A, whereas mice in group B were housed in individual metabolism cages throughout the study period to facilitate complete urine collection.

In group A, blood samples (40—60 μl) were removed from the tail vein at 1, 2, 4, 6, 8, 10, 12, 24, 26.5, 31 h into heparinized micro haematocrit tubes of 20-μl capacity (Hawksley, Lancing, U.K.) which were then heat-sealed. Following centrifugation (11,600 *g*, 4 min) volumes of plasma (20—40 μl) were accurately measured using a 50-μl capacity syringe (Hamilton, Reno, NV, U.S.A.) and placed in microcap tubes. Total urine was collected serially for four days from mice in group B, the volume recorded, and an aliquot placed into a microcap tube. All samples were stored at −20° C until time of analysis.

RESULTS AND DISCUSSION

Chromatograms of extracts of drug free plasma, spiked plasma, and plasma from a mouse dosed with pyrimethamine, are shown in Fig. 2. The corresponding chromatograms of urine extracts are shown in Fig. 3. Pyrimethamine, pyrimethamine 3-N-oxide, and the internal standard, proguanil, were resolved, with retention times of 9.25, 6, and 13.5 min, respectively. The minimum detectable concentration (defined as a peak 3 times baseline noise at 0.0025 a.u.f.s.) in a 20-μl plasma sample was 330 ng/ml for

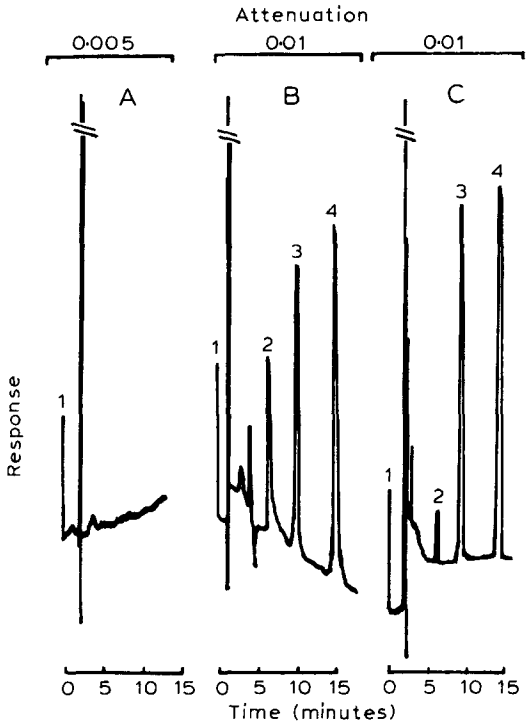

Fig. 2. High-performance liquid chromatograms for (A) a blank plasma extract; (B) a spiked plasma extract (pyrimethamine concentration = 10 μg/ml, pyrimethamine 3-N-oxide concentration = 10 μg/ml); and (C) an extract of a plasma sample taken from a mouse dosed with pyrimethamine at 75 mg/kg (pyrimethamine concentration = 11.7 μg/ml, pyrimethamine 3-N-oxide concentration = 3.0 μg/ml). Peaks: 1 = injection event; 2 = pyrimethamine 3-N-oxide; 3 = pyrimethamine; and 4 = internal standard.

pyrimethamine and 600 ng/ml for the 3-N-oxide metabolite. Analytical recoveries of pyrimethamine, its 3-N-oxide and internal standard were 91.2 ± 5.9%, 82.0 ± 5.1% and 79.0 ± 6.4%, respectively. All calibration curves showed linearity (r = 0.99) for pyrimethamine and pyrimethamine 3-N-oxide in both plasma and urine. The intra- and inter-assay precision data for drug and metabolite in both plasma and urine are summarized in Table I. There was little variation in pyrimethamine assays, with coefficients of variation below 9%. By contrast coefficients of variation of pyrimethamine 3-N-oxide assays were slightly greater at the low concentrations tested.

The assay was shown to be selective, as it was free from chromatographic interference from endogenous material and the antimalarial drugs chloroquine, amodiaquine, cycloguanil, primaquine, sulphadiazine and sulphadoxine; although it was noted, that with some batches of the ODS column used, the resolution between chloroquine and pyrimethamine was incomplete. Therefore, the proposed method would be well suited for pyrimethamine determination, where these other antimalarials were being co-administered, as in combination therapy or studies of drug metabolism interactions.

Sensitive gas—liquid chromatographic (GLC) methods have previously been reported [5, 6]. However they would not be able to quantify N-oxide metab-

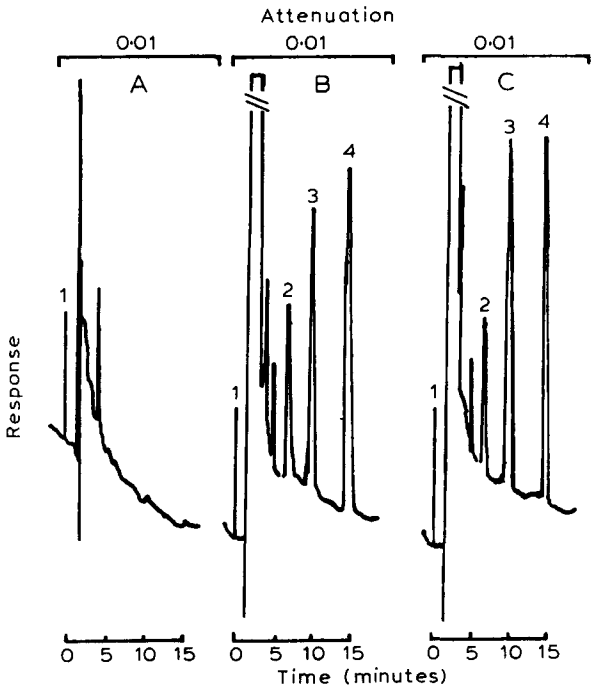

Fig. 3. High-performance liquid chromatograms for (A) a blank urine extract; (B) a spiked urine extract (pyrimethamine concentration = 10 μg/ml, pyrimethamine 3-N-oxide concentration = 10 μg/ml); and (C) an extract of a urine sample from a mouse which received pyrimethamine at 75 mg/kg (pyrimethamine concentration = 12.3 μg/ml, pyrimethamine 3-N-oxide concentration = 8.1 μg/ml). Peaks: 1 = injection event; 2 = pyrimethamine 3-N-oxide; 3 = pyrimethamine; and 4 = internal standard.

TABLE I

INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION FOR PYRIMETHAMINE AND ITS 3-N-OXIDE (n = 5)

Compound	Plasma			Urine		
	Concentration (μg/ml)	Intra-assay C.V. (%)	Inter-assay C.V. (%)	Concentration (μg/ml)	Intra-assay C.V. (%)	Inter-assay C.V. (%)
Pyrimethamine	10	4.8	8.5	100	2.7	3.8
	2	3.0	8.0	10	7.0	5.4
Pyrimethamine 3-N-oxide	5	5.0	2.1	10	4.8	11.0
	1	6.0	10.0	2	7.0	11.9

olites of pyrimethamine, as the thermal instability of these molecules [7] makes them unsuitable for GLC analysis. Furthermore, as N-oxides readily revert to the parent drug at elevated temperatures [8], the accuracy of pyrimethamine determination in such methods may be compromised. These disadvantages do not apply to the HPLC method described in this report.

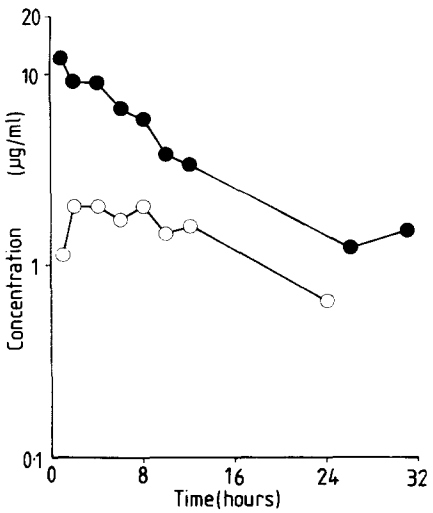

Fig. 4. Representative semi-logarithmic plot of plasma levels of pyrimethamine (•—•) and pyrimethamine 3-N-oxide (○—○) against time, obtained from a single mouse following 75 mg/kg pyrimethamine.

In the present study the assay was used to examine the pharmacokinetics of pyrimethamine in the mouse, which is a convenient and widely used animal model for the study of malaria. A typical log plasma concentration versus time curve is shown in Fig. 4. Plasma levels of pyrimethamine were observed to reach a maximum level of 12.3 μg/ml at 1 h after dosing, after which they decayed with a terminal phase half-life of 7.9 ± 5.9 h, which is in broad agreement with the only previous report [9]. Pyrimethamine 3-N-oxide attained a peak plasma level of 2 μg/ml at 2 h, falling below detectable levels by 26.5 h. Urinary excretion of pyrimethamine over 96 h was 41.8 ± 22.2 μg or 2.3% of the dose. Excretion of pyrimethamine 3-N-oxide was 7.0 ± 5.0 μg or 0.4% of the dose.

The proposed assay has been shown to be suitably selective and sensitive for the quantitation of both pyrimethamine and pyrimethamine 3-N-oxide. As a microanalytical method, it is well suited to analysis of small volumes of plasma and urine, such as those obtained in the chemotherapy of experimental rodent malaria.

ACKNOWLEDGEMENTS

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