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# DETERMINATION OF PYRIMETHAMINE IN HUMAN PLASMA AFTER ADMINISTRATION OF FANSIDAR OR FANSIDAR—MEFLOQUINE BY MEANS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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## SUMMARY

A sensitive, rapid and selective high-performance liquid chromatographic (HPLC) method has been developed to measure plasma levels of pyrimethamine in human subjects dosed with the antimalarials Fansidar or Fansidar and mefloquine. The drug was extracted from plasma at basic pH with *n*-butyl chloride—dichloromethane (96:4, v/v) and quantified on a normal-phase HPLC column with fluorescence detection (excitation 290 nm, emission 345 nm).

Pyrimethamine was almost quantitatively extracted from plasma in the concentration range 20—200 ng/ml. The sensitivity limit was about 10 ng/ml of plasma, using a 0.5-ml specimen. The method was shown to be specific with respect to the other two components in the antimalarial combinations, namely sulfadoxine and mefloquine, and their metabolites.

The assay was applied to pharmacokinetic studies of pyrimethamine in man following the oral administration of Fansidar or Fansidar and mefloquine.

## INTRODUCTION

The emergence of plasmodial resistance to many currently applied antimalarials is becoming a severe problem all over the world. In South East Asia and Latin America especially, the efficacy of chloroquine-related drugs is much less than in previous years. The development of plasmodial resistance can be delayed by the administration of combinations rather than single drugs.

Fansidar, which is used in this study, is an effective antimalarial combination of pyrimethamine and sulfadoxine (weight ratio 1:20). These two drugs inhibit two different enzymes in the plasmodial folate biosynthesis pathway (dihydrofolate reductase and dihydropteroate synthetase). The synergistic

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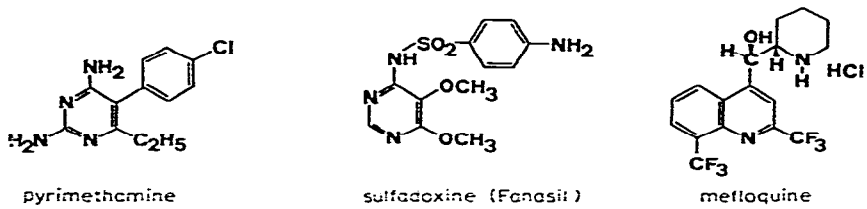


Fig. 1. Chemical structure of pyrimethamine, sulfadoxine and mefloquine.

antiplasmodial effect of pyrimethamine and sulfadoxine can be enhanced by the additive effect of mefloquine, which is a quinoline methanol derivative (for structures see Fig. 1). The use of this triple combination results in a marked delay of resistance development and, because lower doses may be given, in better tolerance and fewer side-effects.

For pharmacokinetic studies, a rapid, specific, and sensitive method was required for the determination of plasma concentrations of pyrimethamine. However, the simultaneous occurrence of the antimalarial components and their respective metabolites in the plasma makes the analytical determination of pyrimethamine difficult, especially as the three components appear in plasma concentrations which differ by a factor of several magnitudes.

A number of methods for the determination of pyrimethamine and related diaminopyrimidines in body fluids and tissues are available, such as microbiology [1], photometry [2], thin-layer chromatography [3—5], gas chromatography [6—8] and liquid column chromatography (with UV detection) [9, 10].

In this paper, we describe a new, normal-phase high-performance liquid chromatographic (HPLC) method for the quantitation of pyrimethamine in plasma without interference from sulfadoxine, mefloquine, or their respective metabolites, combining a rapid single-step extraction with selective and sensitive fluorescence detection.

## EXPERIMENTAL

### *Reagents and solvents*

Sodium hydroxide (p.a.), dichloromethane (for pesticide residue analysis), methanol (for fluorescence spectroscopy), diisopropyl ether (p.a.), aqueous ammonia solution (25%, p.a.) and 5-μm LiChrosorb Si 60 were obtained from E. Merck, Darmstadt, G.F.R. *n*-Butyl chloride (p.a.) was purchased from Fluka, Buchs, Switzerland. Acetonitrile was HPLC-grade S (Rathburn, Walkerburn, Great Britain). Pyrimethamine was of pharmaceutical grade (F. Hoffmann-La Roche & Co., Basle, Switzerland).

Diisopropyl ether, *n*-butyl chloride and acetonitrile were redistilled prior to use. The redistilled diisopropyl ether and the aqueous ammonia solution were stored in the refrigerator.

For preparation of plasma standards, sodium citrated human blood was received from a blood bank (Blutspendezentrum SRK, Basle, Switzerland) and centrifuged at approx. 1000 *g* for 20 min. The blank plasma obtained was tested for the absence of endogenous components interfering with the pyrimethamine and stored at —20°C. In pharmacokinetic studies volunteer blood

samples (10 ml) were collected using vacutainers containing potassium ammonium oxalate as anticoagulant. The plasma was prepared and stored as described above.

*Plasma standards*

Pyrimethamine (10 mg) was dissolved in 10 ml of methanol by ultrasonication to yield the stock solution. The working solutions a—f containing 3200, 1600, 800, 400, 200 and 100 ng of pyrimethamine per 0.1 ml were obtained by diluting aliquots of the stock solution with methanol. The plasma standards A—F containing 320, 160, 80, 40, 20 and 10 ng of pyrimethamine per ml were prepared by diluting 0.1 ml of the corresponding working solution with human blank plasma to 10 ml.

A batch of quality control samples containing 200 ng of pyrimethamine per ml was prepared by diluting 0.5 ml of a methanolic working solution (20 μg/ml) with human blank plasma to 50 ml.

To obtain optimum control of the assay, plasma standards and quality control samples were prepared by different persons using different stock solutions.

The stock solution could be stored in a refrigerator for about four weeks. The working solutions were prepared prior to use. The plasma standards and quality control samples were divided into aliquots of 2.5 ml and stored deep-frozen (—20° C) until required for analysis.

*Chromatographic system*

A modular HPLC system was used, consisting of an Altex pump 110 A (Altex Scientific, Berkeley, CA, U.S.A.), a Rheodyne injector 7125 with a 50-μl loop (Rheodyne, Berkeley, CA, U.S.A.), a Labotron spectrofluorometer SFM 22 (Kontron, Zürich, Switzerland), excitation wavelength 290 nm, emission wavelength 345 nm, a recorder W+W Model 1100 (Kontron) range 50—100 mV, chart speed 0.5 cm/min, and a stainless-steel column, 25 cm × 3.2 mm I.D., containing 5-μm LiChrosorb Si 60. The isocratic mobile phase used was a mixture of methanol—acetonitrile—aqueous ammonia solution (25%)—diisopropyl ether (6:25:0.1:71, v/v). Except for the aqueous ammonia solution, all solvents were degassed before mixing by ultrasonication for about 5 min.

*Procedure*

*Extraction.* An aliquot of 0.5 ml of sample* was added to a ground-glass stoppered centrifugation tube and mixed with 0.5 ml of bidistilled water and 0.2 ml of 2 *N* sodium hydroxide solution. After the addition of 7 ml of *n*-butyl chloride—dichloromethane (96:4, v/v), the sample was extracted by shaking for 15 min at 15 r.p.m. on a rotating shaker (Heidolph) and centrifuged (1700 *g*) at 10°C for 10 min. An aliquot (6 ml) of the organic phase was transferred to a conical glass tube and evaporated to dryness at 40° C by means of a

*Either plasma standard (calibration), control sample (quality control), biological sample (analyses), drug-free plasma (plasma blank), or bidistilled water (reagent blank).

gentle stream of pure nitrogen. The tube containing the remaining extraction residue was stored in the refrigerator until required for analysis.

*Chromatography.* The residue of the extraction was reconstituted with 100 μl of the mobile phase and an aliquot (50 μl) of the clear solution was injected for HPLC analysis.

The flow-rate of the mobile phase was adjusted to 0.7 ml/min, effecting a back pressure of approximately 70 bar. The column was reconditioned overnight with a purge solvent mixture consisting of methanol—acetonitrile—diisopropyl ether (6:25:71, v/v), filtered through a 0.5-μm filter (Millipore) prior to use. The flow-rate of the purge solvent was decreased to 0.2 ml/min.

*Calibration and quality control.* Along with the biological samples, four specimens of standard plasma A—F in the expected concentration range were analysed daily. An external standard curve was obtained by a least-squares regression of the peak heights measured versus the concentrations of pyrimethamine added to the plasma. This calibration curve was then used to interpolate concentrations of pyrimethamine in biological samples from peak height measurements.

Each day a quality control sample containing 200 ng of pyrimethamine per ml was carried through the procedure. Results from the biological samples were accepted provided the calculated concentrations of the quality control samples lay within the range 180—220 ng of pyrimethamine per ml.

## RESULTS

### *Separation*

When operating under the chromatographic conditions described above, the retention time of pyrimethamine was approximately 4.6 min.

### *Sensitivity and limit of detection*

The detector parameters were optimized to obtain 80% full-scale deflection (50 mV recorder input) for a solution containing 50 ng of pyrimethamine per 50 μl of eluent.

The limit of detection for the method was about 10 ng of pyrimethamine per ml of plasma, using a 0.5-ml specimen. For this minimum detectable concentration a signal-to-noise ratio of ca. 3:1 was observed (Fig. 2b).

### *Selectivity*

Several blank plasma samples from different human subjects were tested for the absence of interfering endogenous plasma components. Fig. 2a shows a typical chromatogram of a blank plasma extract.

The selectivity of the assay with respect to sulfadoxine, mefloquine and their metabolites was investigated by administration of a single oral dose of 500 mg of sulfadoxine or 140 mg of mefloquine, respectively, to two healthy male volunteers. Blood samples were taken up to 48 and 264 h, respectively, after administration and analysed as described. In both cases no peak with a retention time similar to that of pyrimethamine was detectable.

In a third experiment a potential interference of the assay by pyrimethamine metabolites was studied. Following a single oral dose of a Fansidar tablet

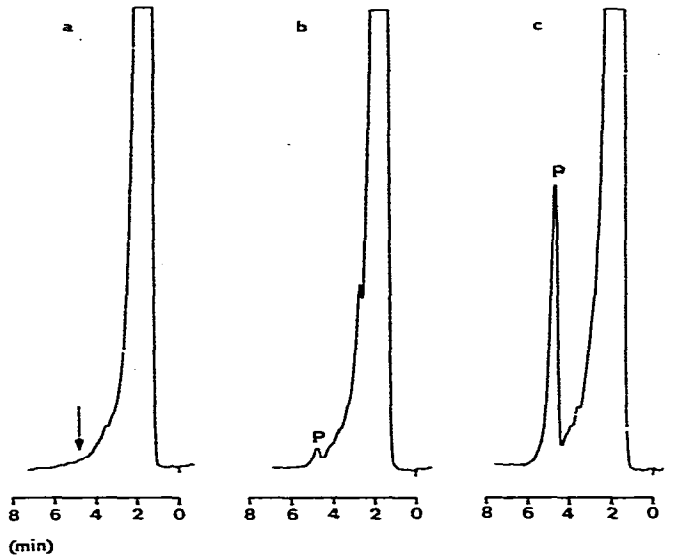


Fig. 2. (a) Chromatogram of a human blank plasma sample. The arrow indicates the retention time of pyrimethamine. (b) Chromatogram of a human blank plasma sample spiked with 10 ng of pyrimethamine (P) per ml. (c) Chromatogram of a human volunteer plasma sample, collected 24 h after a single dose of 40 mg of pyrimethamine (P), 800 mg of sulfadoxine and 280 mg of mefloquine; calculated pyrimethamine concentration 195 ng/ml.

containing 25 mg of pyrimethamine and 500 mg of sulfadoxine, human plasma samples were collected at various times and analysed. No peaks corresponding to possible pyrimethamine metabolites were detectable.

*Linearity*

A linear correlation between peak height and concentration of pyrimethamine was found in the concentration range 10—320 ng/ml of plasma. The coefficient of determination ($r^2$) was generally better than 0.9990 and the intercept did not differ significantly from zero.

*Recovery*

The recovery (extraction yield) was determined by adding known amounts of pyrimethamine to human blank plasma (20 and 200 ng/ml) and analysing each sample in quadruplicate according to the described procedure. Compared to a series of unextracted reference standards the recovery varied between 90 and 98%.

*Reproducibility*

The reproducibility of the external standard method was evaluated over a concentration range of 30.3—303 ng of pyrimethamine per ml. For each concentration a set of five specimens was analysed as described in one day (intra-assay reproducibility).

The inter-assay reproducibility was obtained by analysing one specimen from each concentration on five different days. Additional quality control samples were analysed together with the biological samples on 29 days covering a period of four months.

TABLE I

REPRODUCIBILITY

| Concentration added (ng/ml) | Concentration found (ng/ml) | C.V.* (%) | No. of replicates | Difference between found and added concentration (%) |
|---|---|---|---|---|
| *Inter-assay reproducibility* | | | | |
| 30.3 | 29.4 | 4.7 | 5 | −3.0 |
| 151.5 | 151.9 | 3.2 | 5 | +0.3 |
| 303.0 | 300.0 | 2.4 | 5 | −1.0 |
| 200** | 196.0 | 4.1 | 29 | −2.0 |
| *Intra-assay reproducibility* | | | | |
| 30.3 | 30.0 | 4.3 | 5 | −1.0 |
| 151.5 | 158.0 | 1.4 | 5 | +4.1 |
| 303.0 | 304.2 | 2.2 | 5 | +0.4 |

*Coefficient of variation.
**Quality control sample.

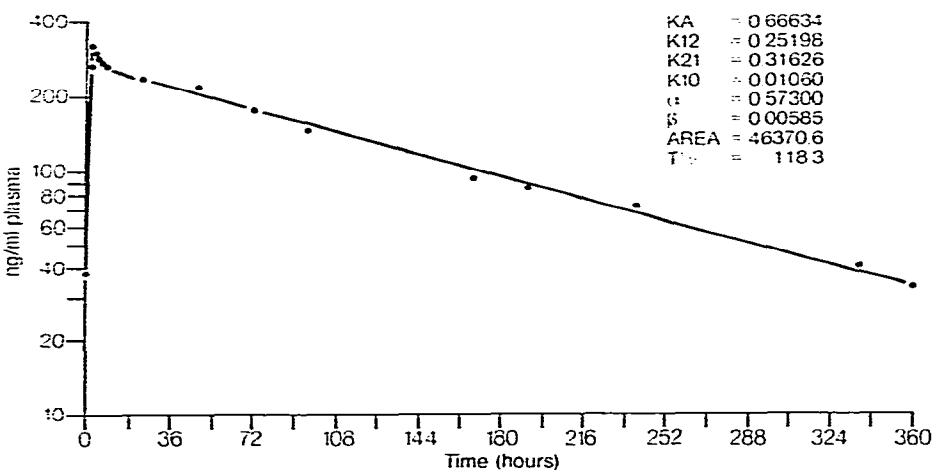


Fig. 3. Pyrimethamine plasma concentrations of volunteer U.T. after administration of two tablets of Ro 13-5112 containing 40 mg of pyrimethamine, 800 mg of sulfadoxine and 280 mg of mefloquine. The calculation of the curve is based on the assumption of a two-compartment model with first-order absorption. Pharmacokinetic data are shown in the inset.

The data presented in Table I demonstrate that the precision (relative standard deviation of replicate analyses) and accuracy (difference between added and found concentration) were acceptable over the concentration range investigated.

*Stability of pyrimethamine in plasma*

Plasma samples containing 320 ng of pyrimethamine per ml were stored for 24 h at ambient temperature and for four months at −20°C. In both cases no significant decomposition of the drug was observed.

*Application of the method to biological samples*

The method has been successfully applied to the analysis of 300 plasma samples from four clinical studies. Fig. 2c shows a typical chromatogram from these studies, demonstrating the validity of the new assay.

A complete plasma profile of one volunteer (U.T.) is given in Fig. 3. Pyrimethamine clearly exhibits two-compartment pharmacokinetics and the plasma concentration—time curve is best fitted by non-linear regression analysis according to $C_{pl} = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} + C \cdot e^{-K_a t}$. The evaluation of the pharmacokinetic data is based on computer programs as described in ref. 11. In the present case we obtained a terminal elimination half-life of 118 h.

## DISCUSSION

The recently published gas chromatographic method of Jones et al. [7] has a sensitivity similar to the method described in this report. However, the analysis time is long (26 min) as a result of long-retained endogenous components. Jones et al. state in their paper that HPLC with fluorescence detection cannot be applied to pyrimethamine on account of its poor fluorescence. We also found that the fluorescent response of pyrimethamine was not as strong as its ultraviolet absorption. However, in our hands, working with a large number of different plasma samples we found fluorescence to be far more specific than ultraviolet absorption. In addition, the sensitivity for this method is as good as that quoted for the methods referred to (10 ng/ml, taking a 0.5-ml sample).

The extraction solvent was varied to obtain a sufficiently high recovery of pyrimethamine without interference from coextracted plasma components. Chloroform, dichloromethane, diethyl ether, benzene and benzene—dichloromethane (9:1, v/v) in some cases gave rise to extraction of endogenous plasma compounds which interfered with pyrimethamine. Less-polar solvents such as heptane and hexane extracted the drug with low efficiency.

An alternative to these "classical extraction solvents" is *n*-butyl chloride, which is being used increasingly for the extraction of basic drugs from biological fluids, especially when using gas—liquid chromatography with nitrogen detection for quantitation [12—15]. We used a mixture of *n*-butyl chloride and dichloromethane (96:4, v/v) and obtained both high recovery of pyrimethamine and clean blank plasma extracts.

Haefelfinger [16] showed recently that the reproducibility of HPLC assays cannot always be improved by means of an internal standard. We investigated two different pyrimethamine analogues as potential internal standards. These substances did not increase the precision of the assay, and for this reason an external standard method was established which showed good reproducibility.

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REFERENCES


1 E. Weidekamm, H. Plozza-Nottebrock, L. Forgo and U.C. Dubach, Bull. World Health Organ., in press.
2 C.C. Peck, A.N. Lewis and B.E. Joyce, Ann. Trop. Med. Parasitol., 69 (1975) 141.
3 C.R. Jones and L.A. King, Biochem. Med., 2 (1969) 251.
4 W.S. Simmons and R.L. DeAngelis, Anal. Chem., 45 (1973) 1538.
5 R.L. DeAngelis, W.S. Simmons and C.A. Nichol, J. Chromatogr., 106 (1975) 41.
6 P. Cala, N.R. Trenner, R.P. Buhs, G. Downing, J. Smith and J.A. Van den Heuvel, J. Agr. Food Chem., 20 (1972) 337.
7 C.R. Jones, P.R. Ryle and B.C. Weatherley, J. Chromatogr., 224 (1981) 492.
8 M. Bonini, F. Mokofio and S. Barazi, J. Chromatogr., 224 (1981) 332.
9 E.M. Levin, R.B. Meyer, Jr. and V.A. Levin, J. Chromatogr., 156 (1981) 181.
10 C.R. Jones and S.M. Ovenell, J. Chromatogr., 163 (1979) 179.
11 C.M. Metzler, G.L. Elfring and A. McEwen, Biometrics, 30 (1974) 562.
12 B.L. Wolen and C.M. Gruber, Anal. Chem., 40 (1968) 1243.
13 W.O. Pierce, T.C. Lamoreaux, F.M. Urry, L. Kopjak and B.S. Finkle, J. Anal. Toxicol., 2 (1978) 26.
14 L. Kopjak, B.S. Finkle, T.C. Lamoreaux, W.O. Pierce, and F.M. Urry, J. Anal. Toxicol., 3 (1979) 155.
15 J.N. Miceli, D.B. Bowman and M.K. Aravind, J. Anal. Toxicol., 5 (1981) 29.
16 P. Haefelfinger, J. Chromatogr., 218 (1981) 73.