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SIMULTANEOUS DETERMINATION OF PYRIMETHAMINE AND MEFLOQUINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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SUMMARY

A rapid, sensitive and selective method was developed for the simultaneous determination of pyrimethamine and mefloquine, two of the active ingredients of Fansimef®, in human plasma The procedure involved extraction of the compounds and the internal standard nitrazepam from basified plasma with dichloromethane and chromatography on a C_{18} column (μBondapak, 300×3 9 mm I D) with acetonitrile–phosphate buffer as the mobile phase and UV detection at 222 nm The limit of quantification was 10 ng/ml for both substances, using a 1-ml plasma specimen The mean inter-assay precision was 2 8% for pyrimethamine and 4 7% for mefloquine up to 800 ng/ml The practicability of the method was demonstrated by the analysis of more than 1200 plasma samples from several pharmacokinetic studies involving single-dose administration of Fansimef to both patients and volunteers

INTRODUCTION

Fansimef® (Hoffmann-La Roche, Basle, Switzerland) is an antimalarial drug consisting of a fixed combination (1 10 20, w/w/w) of pyrimethamine (I), mefloquine (II) and sulphadoxine (III) (Fig 1) The three active ingredients act on protozoa of the *Plasmodium* genus by different mechanisms pyrimethamine, a 2,4-diaminopyrimidine, is a dihydrofolate reductase inhibitor [1], sulphadoxine, a sulphonamide, is a dihydropteroate synthetase inhibitor [2] and mefloquine, a quinolinemethanol, appears to affect membranes of malarial parasites [3] Pyrimethamine and sulphadoxine act synergistically and mefloquine further enhances their effect Fansimef was formulated with the aim of

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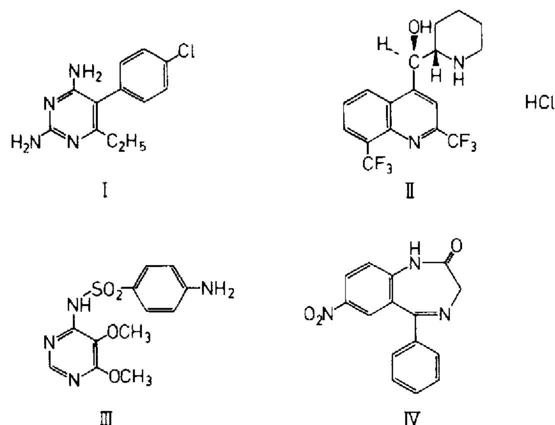

Fig 1 Structures of the components of Fansimef (I = pyrimethamine, II = mefloquine, III = sulphadoxine) and of the internal standard nitrazepam (IV)

reducing the development of resistance to antimalarials by *Plasmodium* strains, and of obtaining better tolerance and fewer side-effects than with Fansidar® (pyrimethamine + sulphadoxine) or Lariam® (mefloquine) alone

For pharmacokinetic studies, a rapid, sensitive and selective method was needed for the determination of plasma concentrations of I and II Several workers have described analytical methods (microbiological [4], thin-layer chromatographic [5–9], gas chromatographic [10–17] and high-performance liquid chromatographic (HPLC) [18–29], furthermore, a review was recently published [30]) for the determination of I or II in body fluids, but no report has appeared so far which enables simultaneous detection of both substances

In this paper we describe a reversed-phase HPLC method for the quantification of both I and II in human plasma without interference from III, with sufficient sensitivity to follow the kinetics of both compounds after single or multiple administration of Fansimef

EXPERIMENTAL

Reagents and solvents

Dichloromethane (Ruckstandsanalyse), methanol (Ruckstandsanalyse), 25% ammonia solution (Suprapur®), potassium dihydrogenphosphate (p a) and orthophosphoric acid (85% reinst) were obtained from E Merck (Darmstadt, F R.G). Acetonitrile (RS for HPLC) and 1 *M* hydrochloric acid (Normex) were from Carlo Erba (Milan, Italy) Water was delivered from a Milli-Q system (Millipore, Milford, MA, U S A) fed with deionized water Pyrimethamine (Ro 4-1558), mefloquine hydrochloride (Ro 21-5998) and nitrazepam (Ro 5-3059) were all of pharmaceutical grade from Hoffmann-La Roche All compounds were used as received

Ammonia-ammonium chloride buffer, pH 9 4 A 38 9-ml volume of 1 *M* ammonia solution and 20 0 ml of 1 *M* hydrochloric acid were diluted to 100 ml with water ($I=0$ 2)

0 1 M Phosphate buffer, pH 3 0 A 13 6-g amount of potassium dihydrogenphosphate was dissolved in about 800 ml of water and adjusted to pH 3 0 with 1 *M* orthophosphoric acid, after filtering through a 0 45-μm membrane (HATF; Millipore) the solution was diluted to 1000 ml with water

Preparation of plasma standards

Stock solutions of I and II were obtained by dissolving 10 and 10 97 mg (equivalent to 10 mg of base), respectively, in 10 ml of methanol A precursor of the methanolic working solutions (solution A) was obtained by mixing 1 6 ml of each stock solution and diluting to 10 ml Working solutions B–F, containing 16, 6, 2, 0 6 and 0 2 μg/ml of both I and II, were obtained by diluting aliquots of solution A with methanol The working solutions could be stored at 25°C for at least 6 months

On every analysis day, calibration samples were prepared by adding 50 μl of the working solutions B–F to 1-ml control plasma specimens to obtain a calibration graph in the range 800–10 ng/ml plasma

For the preparation of plasma standards, fresh frozen human plasma, received from a blood bank (AVIS, Milan, Italy), was thawed at room temperature and centrifuged at 1000 *g* for 2 min After testing for absence of interfering endogenous components, it was stored in 5 5-ml aliquots at −20°C

Quality control samples

Quality control (QC) samples containing 320 and 16 ng/ml of both I and II were prepared A 50-μl volume of a solution containing 128 μg of I and 140 4 μg of II (equivalent to 128 μg of base) per millilitre of methanol was diluted to 20 ml with pretested control plasma (QC 2), and 1 ml of QC 2 was diluted to 20 ml with control plasma to give QC 1 QC samples were divided into 2 2-ml aliquots and stored deep frozen (−20°C) until required for analysis

To obtain an optimum control of the assay, working solutions and QC samples were prepared by different analysts using different stock solutions

Instrumentation

The HPLC system consisted of a T-414 single-piston pump equipped with a Model 812 pulse damper (both from Kontron, Zurich, Switzerland), an automatic sample injector (WISP 710B, Waters Assoc , Milford, MA, U S A), a guard column (RP-2, 30×4 6 mm I D , particle size 10 μm, Brownlee Labs , Santa Clara, CA, U S A), an analytical column (μBondapak C_{18}, 300×3 9 mm I D , particle size 10 μm, Waters Assoc), a variable-wavelength UV detector (Model 4020, Pye Unicam, Cambridge, U K) set at 222 nm and 0 02 a u f s (time constant 1 s) and a strip-chart recorder (Model 8252, Pye Unicam)

The pump delivered the mobile phase, consisting of acetonitrile–0 1 *M* phosphate buffer (pH 3 0) (35 65, v/v) at a flow-rate of 1 5 ml/min (approximately 120 bar back-pressure), the mobile phase (500 ml) was recycled for 1 week or 100 injections The system was stored in acetonitrile–water (35 65, v/v) over weekends. The guard column was replaced after 250 injections or when a pressure increase was observed

The retention times of I, the internal standard nitrazepam (IV) and II were 3 8, 9 5 and 12 4 min, respectively. The analysis time was 14 min

Extraction procedure

Plasma samples were thawed at room temperature, vortex mixed for 10 s and centrifuged at 1000 *g* for 2 min A 1-ml aliquot of plasma sample (calibration standard, QC or unknown) was mixed with 1 ml of ammonia–ammonium chloride buffer (pH 9 4) in a 12-ml conical tube After thorough mixing, 50 μl of a methanolic solution containing 5 μg/ml IV (internal standard) were added, followed by 7 ml of dichloromethane Extraction was performed on a rotating (head-to-head) shaker for 30 min at 25 rpm, followed by centrifugation at 1500 *g* and 4°C for 5 min The aqueous layer was discarded by suction and 5 ml of the organic phase were transferred into a 7-ml conical tube that had previously been silanized with Aquasil (Pierce, Rockford, IL, U S A) and rinsed with methanol, the extract was evaporated to dryness at 45°C under a gentle stream of dry nitrogen

The residue was stored overnight at −20°C, then dissolved in 150 μl of mobile phase by vortex mixing and sonication After centrifugation at 3000 *g* for 1 min, 100 μl were injected automatically Injection was carried out within 1 h of reconstitution, as the internal standard degraded at acidic pH

Calibration and calculation

Five calibration samples covering the expected concentration range were processed daily together with QC and unknown samples, as described above The calibration graph was obtained by weighted linear least-squares regression (weighting factor $1/y^2$) of measured I (or II)/IV (y) peak-height ratios versus the concentration of I (or II) added to plasma (x) The two graphs were then used to interpolate concentrations of I and II in QC and unknown samples from the measured I (or II)/IV peak-height ratios Acquisition and on-line treatment of the data were performed by means of an SP 4200 computer integrator (Spectra-Physics, San José, CA, U S A), working with a custom-written BASIC program (an improved version of that previously described for the SP 4100 [31])

RESULTS AND DISCUSSION

Chromatography

Several mixtures of methanol or acetonitrile with water or phosphate buffers differing in pH, and several brands of C_{18} columns (Waters Novapak C_{18} and

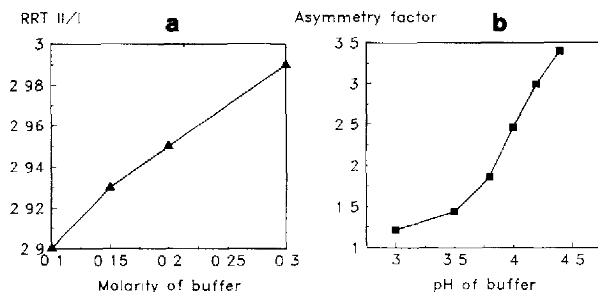

Fig 2 (a) Effect of the molarity of phosphate buffer (pH 3 0) on the relative retention time (RRT) II/I, (b) effect of the pH of 0 1 *M* phosphate buffer on the symmetry of the mefloquine (II) peak

μBondapak C_{18}, Supelco Supelcosil LC-18 and LC-18-DB, Chrompack ChromSpher C_{18}, Merck LiChrosorb RP-18) were tested in order to ensure elution of II, a lipophilic substance, within times compatible with good sample throughput, μBondapak columns and an acetonitrile–phosphate buffer (35 65, v/v) as mobile phase proved to be the best combination Both molarity and, to a greater extent, the pH of the buffer have a critical influence on the retention time and peak shape of II the more acidic the pH, the more symmetric the peak and the higher the molarity, the shorter is the retention time (Fig 2)

The detection wavelength was set at 222 nm, corresponding to the UV maxima of both I and II, in order to obtain high sensitivity

Sample preparation

Several extraction conditions suggested in the literature were tested, however, these were either too cumbersome for routine analysis, requiring several steps or back-extractions, or plasma gave interfering peaks under the chromatographic conditions chosen Basic extraction conditions and high buffering strength were indicated, as III and 2,8-bis(trifluoromethyl)quinoline-4-carboxylic acid, the main metabolite of II, were present as undesirable components in unknown samples, further, an organic solvent showing good selectivity towards I and II was needed Ammonia–ammonium chloride buffer and dichloromethane represented the best compromise between cleanliness of the extract and optimum recovery of the substances (Fig 3)

The choice of the internal standard was affected by two requirements, quantitative recovery and a suitable retention time in the chromatogram, IV was the only compound, among those tested, which satisfied both It is well known [32] that 1,4-benzodiazepines undergo degradation to the corresponding 2-aminobenzophenones under acidic conditions, and IV is no exception (Fig 4) However, it was found that IV was reasonably stable in the mobile phase for at least 1 h, therefore, this was the time limit set between sample reconstitution and injection If recycling of the mobile phase is not performed, it is

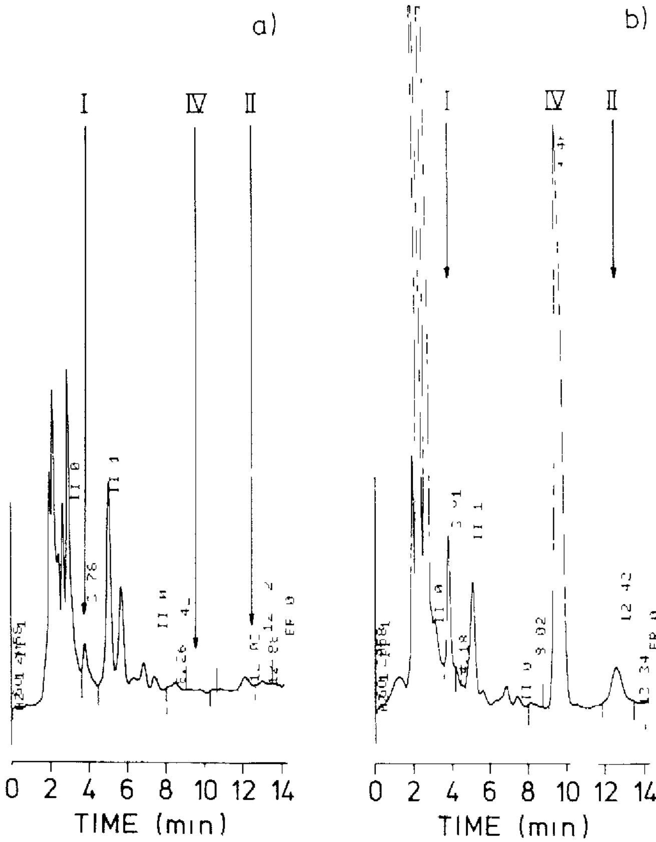

Fig 3 HPLC of human plasma extracts (a) blank plasma from volunteer E B , (b) control plasma spiked with 16 ng/ml of both I and II (QC 1) HPLC conditions as in text, chart speed, 0 5 cm/min

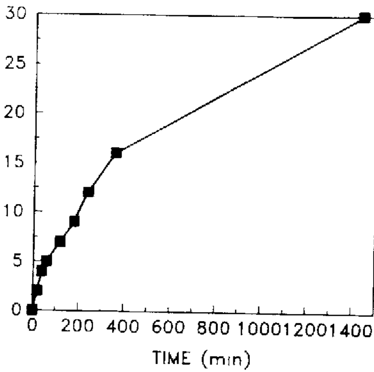

Fig 4 Stability in acetonitrile-0 1 *M* phosphate buffer (pH 3 0) (35 65, v/v) of the internal standard nitrazepam (IV)

TABLE I

RELATIVE RETENTION TIMES (RRT) OF SOME COMMON PHARMACEUTICALS

RRT = Retention time of substance/retention time of pyrimethamine

Compound	RRT	Compound	RRT
Chloroquine	0 53	Sulphamethoxazole	1 40
Caffeine	0 55	Midazolam	1 41
Quinine	0 69	Flurazepam	1 44
Acetaminophen	0 69	Bromazepam	1 48
Sulphadiazine	0 84	Sulphaisoxazole	1 52
Primaquine[a]	0 98	Sulphadimethoxine	1 80
Pyrimethamine	1	Sulphaphenazole	2 02
Diazepam[a]	1 04	Nitrazepam	2 60
Salicylic acid	1 12	Clonazepam	3 11
Acetylsalicylic acid	1 18	Mefloquine	3 30
N^4-Acetylsulphadoxine	1 23	Flunitrazepam	3 99
Sulphadoxine	1 30	2,8-Bis(trifluoromethyl)quinoline-4-carboxylic acid	5 70
N^4-Acetylsulphamethoxazole	1 35		

[a]Interfering substance

possible to overcome all degradation problems by redissolving the residue in acetonitrile–water (35 65, v/v), in which IV is stable

The sample throughput was 25 unknown, 5 calibration and 4 QC samples per 8-h working day

Selectivity

More than 50 blank plasma samples from different volunteers were analysed as described above In all instances, clean extracts in the chromatographic regions of interest were obtained, indicating specificity of the assay with respect to other endogenous components in human plasma

Several common drugs were also tested for interference with I, II and IV under the chromatographic conditions chosen (Table I) Interference was found for primaquine and diazepam only

Limit of quantification, limit of detection

The limit of quantification of the assay, defined as the minimum concentration that could be measured with a precision [coefficient of variation (C V)] better than 10% [33], was found to be 10 ng/ml for both I and II

The limits of detection, defined as the concentration giving a signal-to-noise ratio of 3 1, were 2 and 5 ng/ml for I and II, respectively, using the chromatographic and instrumental parameters defined above

TABLE II

RECOVERIES OF I, II AND IV

Compound	Concentration (ng/ml)	n	Recovery (mean ± C V) (%)
I	10	5	111 4 ± 4 8
	100	5	100 0 ± 0 8
	800	5	100 9 ± 1 5
		Mean	104 1 ± 6 1
II	10	5	107 5 ± 4 5
	100	5	93 1 ± 1 4
	800	5	98 5 ± 1 7
		Mean	99 7 ± 7 3
IV	250	10	103 7 ± 2 8

Recovery

The recovery (extraction yield) of I, II and IV was almost quantitative (Table II) The recovery was determined by comparison, over 3 days, of the substance/internal standard peak-height ratio obtained when the substance was added to plasma and the substance/internal standard peak-height ratio obtained when the substance was added to the plasma extract, corrected for the dilution factor of 5 7, the internal standards were IV for I and II and I for IV

Linearity

A linear correlation between the peak-height ratio of I (or II) to IV and the concentration of I (or II) was found in the range 10–800 ng/ml of plasma This wide range of concentrations (a factor of 80) necessitated the use of a weighting factor in order to avoid division into sub-ranges The coefficient of determination was generally better than 0 9990 for I and 0 9980 for II, and the mean deviation [(added – found)/added] from the calibration graph was better than 2% for I and 3% for II

Precision

The precision of the method was evaluated by replicate analysis of spiked quality control plasma samples The intra-assay data were obtained by replicate analysis of QC plasma samples on the same day The inter-assay data were obtained by analysing the same QC plasma samples on various days, a first set of data was obtained over a time period of 2 weeks and a second set was ob-

TABLE III

PRECISION OF THE HPLC METHOD

Compound	Concentration added (*A*) (ng/ml)	Concentration found (*B*) (ng/ml)	*n*	C V (%)	Relative error [100(*B*−*A*)/*A*] (%)
Intra-assay precision					
I	16 0	16 5	6	2 2	+3 1
	320 0	315 8	6	0 8	−1 3
II	16 0	17 3	6	2 8	+7 9
	320 0	338 4	6	0 9	+5 8
Inter-assay precision (2 weeks)					
I	16 0	16 3	10	4 6	+1 7
	32 0	31 7	10	2 2	−1 0
	320 0	302 8	10	1 7	−5 4
II	16 0	16 6	10	8 1	+3 9
	32 0	31 3	10	3 4	−2 3
	320 0	299 1	10	2 6	−6 5
Inter-assay precision (4 months)					
I	16 0	16 0	94	6 3	+0 3
	320 0	318 4	96	3 0	−0 5
II	16 0	16 4	94	9 2	+2 5
	320 0	325 4	96	4 2	+1 7

tained during routine analysis over 4 months, analysis was carried out by four analysts on two different columns from different batches The data shown in Table III demonstrate the good precision of the method over the concentration range investigated No significant time- or column-dependent influence on the inter-assay precision data could be observed

Application of the method to biological samples

The method has been applied successfully to the analysis of about 1200 plasma samples, mostly from a bioequivalence study in man between two different dosage forms Fig 5 shows representative chromatograms from this study The method was sensitive enough to measure the small concentrations of I and II in plasma for up to 300 and 1344 h, respectively (corresponding to approximately four elimination half-lives [34]) after a single oral dose of one tablet of Fansimef (25 mg of I, 250 mg of II and 500 mg of III) to human volunteers (Figs 6 and 7) The method also permits the assessment of pharmacokinetic parameters from plasma concentration–time data after doses corresponding to the usual therapeutic regimen (one tablet weekly)

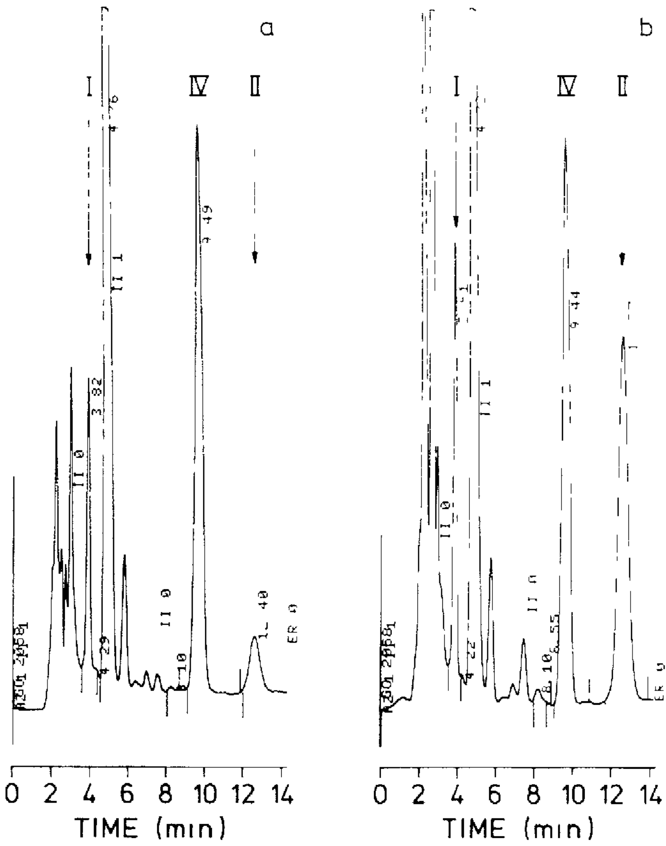

Fig 5 (a) Plasma from volunteer E B , 0 5 h after administration of one tablet of Fansimef (25 mg of I, 250 mg of II, 500 mg of III), calculated concentrations 40 46 ng/ml for I and 27 99 ng/ml for II, (b) plasma from the same volunteer after 120 h, calculated concentrations 61 16 ng/ml for I and 178 ng/ml for II HPLC conditions as in text, chart speed 0 5 cm/min

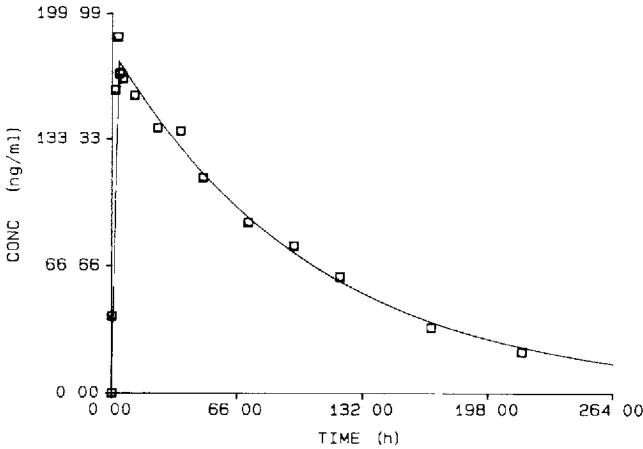

Fig 6 Plasma concentration-time course of I following a single oral dose of one tablet of Fansimef (25 mg of I, 250 mg of II, 500 mg of III) to a healthy volunteer (E B)

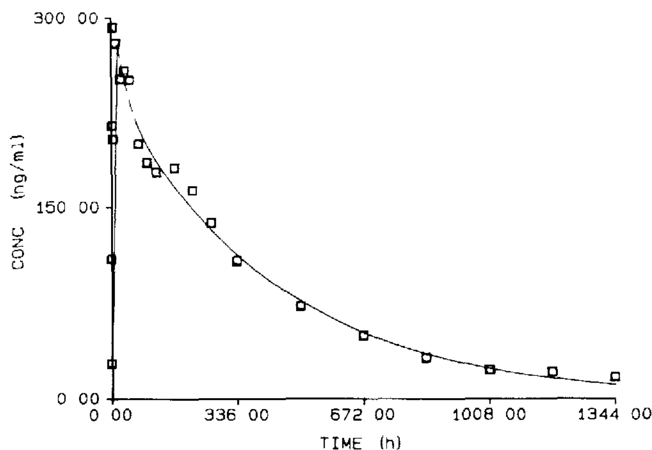

Fig 7 Plasma concentration-time course of II following a single oral dose of one tablet of Fansimef (25 mg of I, 250 mg of II, 500 mg of III) to a healthy volunteer (E B)

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REFERENCES

1 R Ferone, J J Buchall and G H Hitchings, Mol Pharmacol, 5 (1969) 49
2 I M Rollo, Br J Pharmacol, 10 (1955) 208
3 R E Brown, F A Stancatto and A D Wolfe, Life Sci, 25 (1979) 1857
4 E Weidekamm, H Plozza-Nottebrock, I Forgo and U C Dubach, Bull WHO, 60 (1982) 115
5 C R Jones and L A King, Biochem Med, 2 (1969) 251
6 W S Simmons and R L DeAngelis, Anal Chem, 45 (1973) 1538
7 R L DeAngelis, W S Simmons and C A Nichol, J Chromatogr, 106 (1975) 41
8 R A Ahmad and H J Rogers, Br J Clin Pharmacol, 10 (1980) 519
9 D E Schwartz, in A Frigerio and M McCamish (Editors), Recent Developments in Chromatography and Electrophoresis, 10, Elsevier, Amsterdam, 1980, p 80
10 T Nakagawa, T Higuchi, J L Haslam, R D Shaffer and D W Mendenhall, J Pharm Sci, 68 (1979) 718
11 D E Schwartz and U B Ranalder, Biomed Mass Spectrom, 8 (1981) 589
12 M Bonini, F Mokofio and S Barazi, J Chromatogr, 224 (1981) 332
13 C R Jones, P R Ryle and B C Weatherley, J Chromatogr, 224 (1981) 492
14 C Midskov, J Chromatogr, 306 (1984) 388
15 P Heizmann and R Geschke, J Chromatogr, 311 (1984) 411
16 D Dadgar, J Climax, R Lambe and A Darragh, J Chromatogr, 337 (1985) 47
17 Y Bergqvist, F C Churchill and D L Mount, J Chromatogr, 428 (1988) 281

18 J M Grindel, P F Tilton and R D Shaffer, J Pharm Sci , 66 (1977) 834
19 C R Jones and S M Ovenell, J Chromatogr , 163 (1979) 179
20 U Timm and E Weidekamm, J Chromatogr , 230 (1982) 107
21 I M Kapetanovic, J G DiGiovanni, J Bartosevich, V Melendez, J Von Bredow and M Heiffer, J Chromatogr , 277 (1983) 209
22 M Edstein, J Chromatogr , 305 (1984) 502
23 C Midskov, J Chromatogr , 308 (1984) 217
24 M D Coleman, G Edwards, G W Mihaly, R E Howells and A M Breckenridge, J Chromatogr , 308 (1984) 363
25 Y Bergqvist and M Eriksson, Trans R Soc Trop Med Hyg , 79 (1985) 297
26 H S Lee, T Y Ti, P S Lee and C L Yap, Ther Drug Monit , 7 (1985) 415
27 P J Arnold and O V Stetten, J Chromatogr , 353 (1986) 193
28 Y Bergqvist, S Eckerbom, N Larsson and F C Churchill, J Chromatogr , 427 (1988) 295
29 Y Bergqvist, U Hellgren and F C Churchill, J Chromatogr , 432 (1988) 253
30 Y Bergqvist and F C Churchill, J Chromatogr , 434 (1988) 1
31 U Timm, D Dell and D A Ord, Instrum Comput , 3 (1985) 29
32 H Schutz, Benzodiazepines, Springer, Berlin, 1982, p 14
33 M A Brooks and R E Weinfeld, Drug Dev Ind Pharm , 11 (1985) 1703
34 Investigational Drug Brochure of Ro 13-5112, a Fixed Combination of Mefloquine and Fansidar for Suppression of Malaria, Hoffmann-La Roche, Basle, 1982