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Determination of a new antimalarial drug, benflumetol, in blood plasma by high-performance liquid chromatography

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Abstract

A rapid and selective high-performance liquid chromatographic assay for determination of a new antimalarial drug (benflumetol, BFL) is described. After extraction with hexane–diethyl ether (70:30, v/v) from plasma, BFL was analysed using a C_{18} Partisil 10 ODS-3 reversed-phase stainless steel column and a mobile phase of acetonitrile–0.1 *M* ammonium acetate (90:10, v/v) adjusted to pH 4.9 with ultraviolet detection at 335 nm. The mean recovery of BFL over a concentration range of 50–400 ng/ml was 96.8±5.2%. The within-day and day-to-day coefficients of variation were 1.8–4.0 and 1.8–4.2%, respectively. The minimum detectable concentration in plasma for BFL was 5 ng/ml with a C.V. of less than 10%. This method was found to be suitable for clinical pharmacokinetic studies.

Keywords: Benflumetol

1. Introduction

Resistance of *Plasmodium falciparum* to many synthetic antimalarial drugs has become widespread in Southeast Asia, Africa and South America [1,2]. This has triggered a search for new antimalarial drugs and one of these compounds, benflumetol (BFL, Fig. 1), has been demonstrated to have promising antimalarial activity in preclinical efficacy studies and clinical trials in patients with *P. falciparum* infection in China [3]. However, currently no plasma concentration data of BFL in patients during prophylactic and chronic dosage are reported. In addition, clinical pharmacokinetic studies of BFL are in progress in China.

BFL (α-dibutylaminomethyl)-2,7-dichloro-9-(*p*-chlorobenzylidene-4-fluorenemethanol) (Fig. 1) was synthesized in the 1970s by the Academy of Military Medical Sciences, Beijing and registered in China for use as an antimalarial drug in 1987 [3]. The compound is an odourless yellow powder, is poorly soluble in water, oils and most organic solvents, but is soluble in unsaturated fatty acids [3]. In order to elucidate the pharmacokinetic properties of this new compound, it was necessary to develop a reliable assay for BFL in biological fluids. The present report describes a selective and sensitive method for the determination of BFL and this assay method has been applied for measurement of the drug con-

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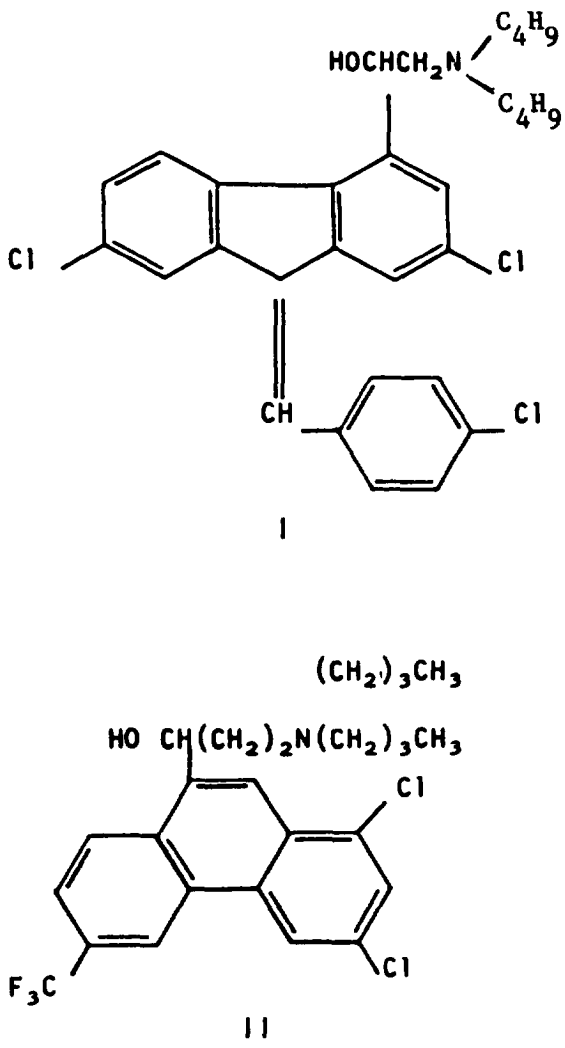

Fig. 1. Structures of benflumetol (I) and halofantrine (II).

centrations in plasma samples obtained from a pilot study involving two healthy Malaysian volunteers.

2. Experimental

2.1. Chemicals

BFL was obtained from Prof. W.H. Wernsdorfer (Institute for Specific Prophylaxis and Tropical Medicine, University of Vienna, Vienna, Austria) and halofantrine (internal standard, Fig. 1) was obtained from Dr. R.J. Horton (SmithKline and Beecham, Hert, UK). All chemicals and solvents were of analytical grade. HPLC-grade acetonitrile, glacial acetic acid, diethylamine, diethyl ether, hexane and methanol were purchased from Merck (Darmstadt, Germany). Dimethylsulphoxide and ammonium acetate were purchased from BDH (Poole, UK). Potassium hydrogen phthalate was obtained from Ajax Chemicals (Auburn, Australia). Dichlorodimethylsilane was purchased from Merck.

2.2. Chromatography

The analytical instrument used was a 501 HPLC pump (Waters, Milford, MA, USA) equipped with a syringe-loading sample injector with a 20-μl sample loop (Model 7125, Rheodyne, Cotati, CA, USA) coupled to a variable-wavelength ultraviolet detector (Hewlett-Packard Series 1050, Avondale, PA, USA) operated at 335 nm. The chromatograms were recorded using an electronic integrator (Model 3392A, Hewlett-Packard). Chromatographic separations were achieved on a C_{18} reversed-phase Partisil 10 ODS-3 stainless-steel column (250×4.6 mm I.D., 10 μm particle size; Phenomenex, Torrance, CA, USA) maintained at room temperature. The mobile phase was acetonitrile–0.1 *M* ammonium acetate (90:10, v/v) adjusted to pH 4.9 with glacial acetic acid at a flow-rate of 1.5 ml/min.

2.3. Extraction procedure

Extraction was carried out in 15-ml glass test tubes pretreated with dichlorodimethylsilane in toluene (5%, v/v) in order to minimise drug adsorption. To a sample of plasma (1 ml) containing a fixed amount of the internal standard, halofantrine (50 μl, 5000 ng/ml) and 0.1 *M* phthalate buffer (1 ml; pH 2.0) were added and the mixture was vortex-mixed for 30 s. The resultant mixture was extracted with 7 ml of hexane–diethyl ether (70:30, v/v) by mechanical tumbling at the speed of 10 rpm for 30 min. A mixture of hexane–diethyl ether needs to be used in order to obtain a good and consistent recovery. After centrifugation (1440 *g*, 15 min) the aqueous phase was frozen in dry ice–methanol mixture and the separated organic phase was evaporated to dryness under a gentle stream of nitrogen at room temperature. In order to obtain a clean chro-

matogram, the residue was reconstituted in 50 μl of methanol–water–glacial acetic acid–diethylamine (93:6:1:0.03, v/v) and 20 μl was injected on to the column for analysis.

2.4. Calibration

Stock solutions of BFL (1.0–20 ng/μl) and the internal standard (100 ng/μl) were prepared by dissolving each substance in dimethylsulphoxide and stored at −20°C. Calibration curves were prepared by spiking drug-free plasma samples with standard solutions 25–800 ng, 25–40 μl) of BFL and the internal standard (5000 ng, 50 μl) to give a concentration range of 25–800 ng/ml for BFL and 5000 ng/ml for the internal standard, halofantrine. The samples were taken through the extraction procedure and the peak-height ratio of the drug was plotted against the corresponding concentration of drug. Linear regression of the peak-height ratio versus the drug concentration ranging from 25 to 800 ng/ml was performed in order to estimate the slope, intercept (peak-height ratio for zero concentration) and correlation coefficient for each standard curve.

2.5. Analytical recovery and assay precision

The analytical recoveries of the extraction procedure for BFL were determined by comparing the peak heights obtained from extracted plasma samples containing known amounts of the substance with those obtained from equivalent amounts of the compound in dimethylsulphoxide by direct injection. The within-day and day-to-day precision were determined at four concentrations by replicate assays of samples from pools of plasma spiked to 50, 100, 200 and 400 ng/ml. The day-to-day assay variation was assessed over a period of five days.

2.6. Healthy volunteers study

Two male volunteers, aged 26 and 31 years and weighing 64 and 80 kg, respectively, were selected for the study. The study protocol was approved by the Institutional Ethics Committee. The investigations were carried-out in accordance with the principles laid down by the World Medical Assembly of 1975 on Ethics in Human Experimentation and informed written consent was obtained from all subjects. No other drugs or any alcohol were taken for seven days prior to or during the clinical trial. Two capsules, each containing 250 mg micronized benflumetol were administered to each subject with 150 ml of water following an overnight fast. A normal breakfast was served 3 h later. Venous samples (10 ml) were taken pre-dose, then after 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48, 72, 120, 168, 216 and 264 h into a lithium heparin vacutainer. Blood was centrifuged (1440 *g* for 20 min) and the plasma was removed and stored at −80°C until analysed. The BFL stability test against temperature (−20°C) in our laboratory showed that only 75% of BFL was recovered after three months of storage. Therefore, storage at −80°C is essential to prevent breakdown of BFL.

2.7. Pharmacokinetic analysis

Data in the text are presented as mean±S.D. values. The elimination half-life was calculated by regression analysis of the log linear portion of the plasma concentration versus time curve. The area under the plasma concentration–time curve (AUC) was calculated by linear trapezoidal rule. Other pharmacokinetic parameters such as plasma clearance (dose/$AUC_{0-\infty}$) and apparent volume of distribution (dose/K $AUC_{0-\infty}$) were calculated using standard model-independent formulae [4]. Maximum concentration and time to reach maximum concentration are the observed values.

3. Results and discussion

The reported sample preparation, which was rapid and simple, permitted the compound of interest to be extracted from plasma without interference from endogenous substances under the given chromatographic conditions. Fig. 2 illustrates the chromatograms obtained typically from drug-free plasma (a), a standard mixture (b), and from a healthy volunteer having received 500 mg of BFL by the oral route (c). The method yields clean chromatograms, with baseline resolution of the internal standard and BFL, at the retention times of 13.3 and 15.9 min, respectively.

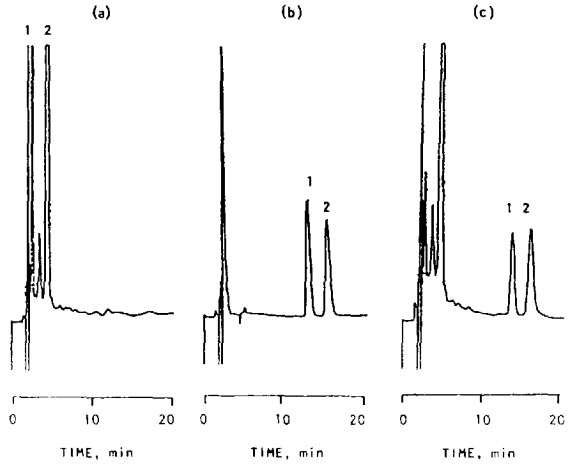

Fig. 2. Chromatograms obtained following (a) extraction of drug-free plasma, (b) a standard mixture in dimethylsulphoxide of BFL (200 ng) and halofantrine (5000 ng) and (c) plasma obtained from a healthy volunteer 2 h after oral administration of 500 mg BFL (concentration=253.1 ng/ml). Peaks: 1=halofantrine (internal standard); 2=BFL. Attenuation: 0.05 AUFS.

Calibration curves were linear (r>0.999) in the range 0–800 ng/ml. The equation of the calibration plots (n=5) for BFL was y=0.0041x−0.0136. y is peak-height ratio of BFL to the internal standards and x denotes various spiked plasma concentrations of BFL (ng/ml). For every batch of analysis a new calibration curve was established. Mean analytical recovery from human plasma was 96.8±5.2% for BFL (Table 1). The minimum detectable concentration of BFL corresponding to a peak three times the baseline noise at 0.005 AUFS was 5 ng using 1 ml of plasma. The within-day coefficients of variation (C.V.) for BFL were 1.8–4.0% (Table 2), and the day-to-day C.V. values were 1.8–4.2% (Table 3).

The validated method for plasma was used to study the pharmacokinetics of the BFL in two healthy volunteers after a single oral 500 mg dose of the micronized drug. The plasma concentration–time profile over the period of 0–264 h is shown in Fig. 3. BFL could be detected at 3 and 1.5 h post-dose in subjects 1 and 2, respectively. Plasma concentrations of BFL were measurable in both subjects throughout the 264 h of the study. In the first subject, the maximum blood concentration (C_{max}) of 18 165 ng/ml was reached 12 h post-dose, and the $AUC_{0-\infty}$ was 561 975 ng h/ml. The plasma clearance (Cl), apparent volume of distribution (V_d) and elimination half-life ($t_{1/2}$) were 14.8 ml/min, 159.2 l and 123.9 h, respectively. With respect to the second subject, BFL reached a C_{max} (17 668 ng/ml) at 6 h post-dose, with an $AUC_{0-\infty}$ of 48 1192 ng h/ml. Cl, V_d and $t_{1/2}$ values in this subject were 17.3 ml/min, 112.1 l and 74.7 h, respectively. A recent clinical study in Chinese volunteers given a single oral dose of 800 mg showed that peak concentrations in plasma were reached after 4–8 h [3]. The mean elimination half-life was 47.4 h. There was marked variation in the pharmacokinetic parameters indicating poor bioavailability of BFL in Chinese subjects [3].

The BFL concentrations and AUC values measured in the Malaysian volunteers were surprisingly high. In the perspective of the poor water solubility of BFL this might indicate very high plasma protein binding of the drug. The time frame for blood sampling was adjusted to the $t_{1/2}$ reported by the Chinese scientists. The elimination half-life in the two Malaysian volunteers was considerably longer,

Table 1
Mean recoveries of BFL (n=5)

Spiked plasma concentration (ng/ml)	Recovery (%)	C.V. (%)
50	90.2	4.3
100	100.2	4.0
200	101.6	2.1
400	95.2	2.8

Table 2
Within-day precision for assay of BFL (n=6)

Spiked plasma concentration (ng/ml)	Mean concentration determined (ng/ml)	C.V. (%)
50	48.3	4.0
100	101.1	3.6
200	201.3	1.8
400	399.3	2.2

Table 3
Day-to-day precision for assay of BFL (n=10)

Spiked plasma concentration (ng/ml)	Mean concentration determined (ng/ml)	C.V. (%)
50	48.2	4.2
100	100.7	3.6
200	202.2	1.8
400	399.3	1.9

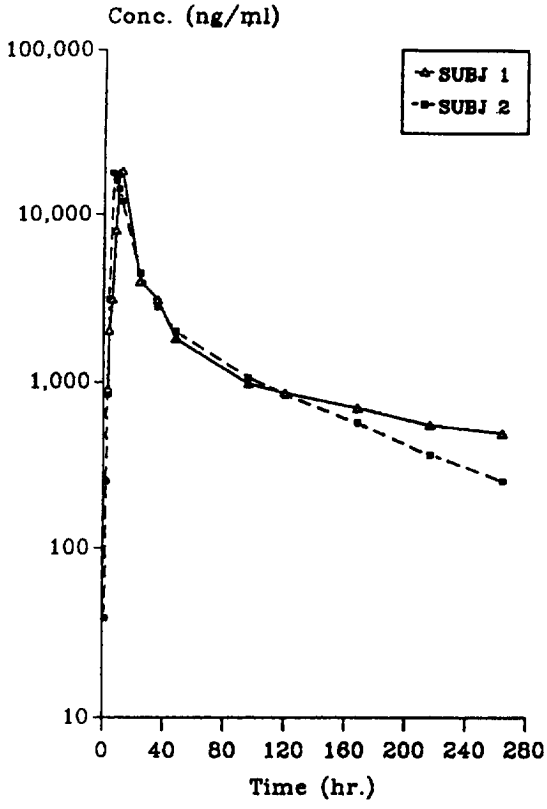

Fig. 3. Plasma concentrations of BFL in two healthy Malaysian volunteers (0–264 h) following the oral administration of a single dose (500 mg) of benflumetol.

so that the AUC and $t_{1/2}$ values would require upward adjustment. For future pharmacokinetic studies of BFL it would be appropriate to add sampling points at 14 and 28 days.

The above described analytical method for the determination of BFL fulfils all the criteria required for an assay to be suitable for clinical pharmacokinetic studies. Its speed, simplicity and low cost make it useful for routine drug analysis. Preliminary phase I pharmacokinetic results imply that there may be pharmacokinetic differences between Chinese and Malaysian volunteers following administration of a single oral dose of benflumetol.

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