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LACK OF EFFECT OF HALOFANTRINE ON HEPATIC DRUG METABOLISM IN THE RAT *IN VIVO* AND *IN VITRO*

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Abstract—The effect of the antimalarial drug halofantrine (Hf) on hepatic drug metabolism in the rat has been studied *in vivo* and *in vitro* using different model drug substrates. Hf *in vitro* produced no significant effect on the values of K_m and V_{max} for aminopyrine N-demethylation or 7-ethoxycoumarin O-dealkylation in microsomes incubated with Hf (0.01–0.1 mM) or on the rate of N-demethylation of aminopyrine or O-dealkylation of Ec in microsomes produced from rats dosed chronically with Hf (200 mg/kg) for 4 days. The disposition of antipyrine (Ap) was investigated in the isolated perfused rat liver preparation (IPRL). Following the administration of bolus doses of Hf (0.5, 2.5 and 5.0 mg) no significant changes were observed in the half-life ($t_{\frac{1}{2}}$), clearance (Cl) or apparent volume of distribution (Vd) for Ap compared with controls. Pentobarbitone induced sleeping time was also assessed in mice. No significant difference was determined in time to recovery of the righting reflex for mice receiving Hf as single oral doses or chronically over 4 days when compared with appropriate controls. The potential for selective isoenzyme effects was studied *in vivo*. The three principal urinary metabolites of Ap, norantipyrine (Np), 3-OH and 4-OH Ap were measured in rat urine, with no significant change in urinary recovery of Ap or any of the metabolites in the presence of Hf (1.25 mg/kg i.p.) compared with controls. These results suggest that Hf is not, in contrast to many commonly used quinoline antimalarials, a potent or specific inhibitor of drug metabolism *in vitro* or *in vivo*.

Several nitrogen heterocyclics including the aminoquinoline antimalarials have been shown to inhibit cytochrome P450 dependent mixed function oxidase activity [1]. Halofantrine, (3-(dibutylamino)-1-[1,3dichloro-6-(trifluoromethyl)-9-phenanthryl] propan-1-ol; Hf) (Fig. 1a) is a phenanthrenemethanol effective *in vivo* against multi-drug resistant strains of *Plasmodium falciparum*. [2–4]. Although not a aminoquinoline, Hf possesses some structural features which are common to compounds of this type, notably a nitrogen-containing an aliphatic alkylamino side chain and a trifluoromethyl substituted phenanthrene nucleus. This side chain is subject to dealkylation (Fig. 1b) accessible sterically and may be able to interact with cytochrome P450 and inhibit drug oxidation. We have used the elimination of antipyrine (Ap) from the isolated perfused rat liver as an index of mixed function oxidase activity. The results obtained from this experimental model, when applied to the study of hepatic drug–drug interactions for the H_2-receptor antagonists [5], have agreed with findings from human studies [6]. More recently this system has been used to investigate the effect of the 8-aminoquinoline antimalarial primaquine, its enantiomers and its metabolites on hepatic drug metabolism [7]. In addition, the model allows the direct evaluation of hepatic drug–drug metabolism interactions in a whole organ system. In further studies we have examined the effect of Hf on *in vitro* microsomal mixed function oxidase activity, on the duration of action of pentabarbitone in mice and the effect on Ap metabolism *in vivo* in an attempt to assess the effect of Hf on hepatic drug metabolizing activity *in vivo* and *in vitro*.

MATERIALS AND METHODS

Drugs and chemicals. Halofantrine hydrochloride (Hf), [^{14}C] ring labelled Hf, (sp. act. 25.82 μCi/mg) and desbutylhalofantrine hydrochloride (Hfm), were gifts from SK and B Research (Welwyn Garden City, U.K.). Antipyrine (Ap), aminopyrine (Am), semicarbazide, norantipyrine (Np), 3-OH and 4-OH antipyrine, phenacetin (Ph), 7-hydroxycoumarin (7-OHc) and 7-ethoxycoumarin (7-Ec) were obtained from the Sigma Chemical Co. (Poole, U.K.). Solvents were of HPLC grade and obtained from Fisons (Loughborough, U.K.). Propane 1–2 diol was obtained from BDH (Poole, U.K.) and pentobarbitone was supplied by May and Baker (Dagenham, U.K.).

Animals. Male Wistar rats (200–250 g) and female CBA mice bred in the Departmental Unit (30–35 g) were housed in well ventilated cages and kept at a temperature of approximately 24°. They were allowed to feed on pelleted food (Oxoid breeding diet, Oxoid Ltd, London, U.K.) and tap water *ad lib*.

Isolated perfused rat livers (IPRL). Rats were anaesthetized with sodium pentabarbitone (60 mg/mL; 60 mg/kg i.p.) and their livers isolated using standard techniques and perfused at 37° in a constant flow (15 mL/min) recirculating system with a 100 mL/reservoir volume as described previously [5]. The principal indices of liver viability were normal visual appearance, steady oxygen consumption, sus-

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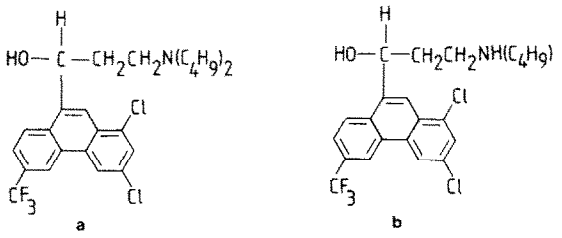

Fig. 1. Structures of (a) halofantrine and (b) desbutyl halofantrine.

tained bile production (0.4–0.6 mL/hr) and constant perfusion pressure (6–8 cm H_2O) over the experimental period of 4 hr.

Antipyrine disposition studies in the IPRL. The elimination of 2.5 mg bolus dose of antipyrine (250 μL of 100 mg/mL aqueous solution) added to the perfusate reservoir was studied over 4 hr in four groups of rats i.e. controls; without the addition of Hf, (N = 5) and after the administration of bolus doses of Hf (0.5, 2.5 and 5.0 mg, N = 5 in each set). All solutions of Hf were prepared in methanol such that the total volume of methanol introduced into the perfusate reservoir was < 0.05 mL. Samples (1.0 mL) were taken from the perfusate reservoir for estimation of antipyrine concentrations, predose and at 5, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min. After centrifugation, (1000 *g*, 10 min) the perfusate plasma was removed and frozen at −70° until assayed. Additional samples (1.0 mL) were taken at 1, 2, 3 and 4 hr for measurement of Hf and Hfm. An equal volume of fresh perfusate was added to the reservoir to replace that removed by sampling. The total amount of antipyrine lost through sampling was < 5% of the dose. Bile was collected into preweighed vials at 0–1, 1–2, 2–3 and 3–4 hr. On termination of each experiment, the liver was frozen immediately in dry ice: methanol and stored at −70°.

Analysis of antipyrine. Perfusate antipyrine concentrations were determined by the HPLC method of Shargel *et al.* [8]. To the perfusate plasma, phenacetin (200 μL) was added as the internal standard, (100 μg/mL, 200 μL), in a 1.5 mL capacity microfuge tube. Perfusate proteins were precipitated by addition of 100 μL each of $ZnSO_4$ (20%), : methanol (50:50 v/v) and saturated $Ba(OH)_2$. After vortex mixing (30 sec) and centrifugation (1000 *g*, 60 sec), 5–10 μL of the supernatant was injected into the chromatograph. Separation was achieved on a μBondapak C18 "Rad Pak" column (Millipore-Waters, Hartford, U.K.) housed in a Z-module (Millipore-Waters). The mobile phase was CH_3OH : NaH_2PO_4 (50 mM, pH 6.8) [40:60] flowing at 3 mL/min. Under these conditions, antipyrine and phenacetin were resolved to baseline and eluted with retention times of 4.0 and 7.5 min, respectively.

Microsomal incubations in vitro. The potential of Hf to inhibit Am N-demethylation and 7-EC O-dealkylation was determined in microsomes prepared as described previously [7] from untreated rats, rats chronically dosed with Hf (20 mg/kg/day i.p. in methanol-100 μL) and rats chronically dosed with vehicle (100 μL/kg/day i.p. for 4 days). Microsomal protein was determined by the method of Lowry *et al.* [9].

N-Demethylation of Am. Am (0.75–5.0 mM), semicarbazide (100 mM), microsomes (3 mg/mL suspension) and NADPH (1.0 mM). Hf (0.01–0.1 mM in methanol) was added as appropriate. Incubation was performed at 37° for 15 min and the reaction terminated by the addition of zinc sulphate 20%: methanol (50:50 v/v; 1 mL) and saturated barium hydroxide (1 mL). Following centrifugation (2000 *g*, 10 min) an aliquot (1 mL) was taken and formaldehyde production assessed using Nash reagent and the absorbance determined at 415 nm [10].

O-Dealkylation of 7-Ec. 7-Ec (10–150 nM in methanol) was evaporated to dryness under nitrogen in glass screw-capped tubes, (Oakes Eddon, Liverpool, U.K.). Hf was added also as a solution in methanol (0.01, 0.05 and 0.1 mM), dried under nitrogen and the residual material dissolved in dimethylsulphoxide, (DMSO; 50 μL). In addition, [1-^{14}C propyl]Hf (20 μL, sp. act. 25.8 μCi/mg) was added to one of the tubes at each concentration of Hf. After sonication (30 min) and prior to incubation an aliquot (20 μL) of the mixture was removed and the amount of radioactivity present in the mixture was determined using liquid scintillation counting. Given the poor solubility of Hf, the aim of this was to determine the actual concentration of Hf which potentially was available for interaction. Phosphate buffer 0.067 M pH 7.5 (1 mL), a microsomal suspension (0.5 mg/mL) and NADPH (1.0 mM) were added and the mixture incubated at 37° for 15 min, in the presence of Hf (0.01–0.1 mM), added previously as appropriate. The reaction was terminated by the addition of trichloroacetic acid 10% (200 μL) and chloroform (2 mL). Following centrifugation (2000 *g*, 10 min) and aspiration the upper layer and the organic phase was (1 mL) transferred to glycine buffer (0.4 M; pH 10.4; 4 mL) contained in clean glass tubes. After centrifugation an aliquot of the aqueous layer assessed for fluorescence at an emission wavelength of 456 nm and an excitation wavelength of 368 nm [11].

Following each incubation the concentration of cytochrome P-450 was determined using the method of Omura and Sato [12].

Sleeping times. Study (1): Forty-eight female, brown mice (mean weight = 30 g) were used in four experimental groups (N = 12). In the control group, mice were administered the vehicle propane 1-2 diol (10%, 0.2 mL) orally 1 hr prior to anaesthesia. Three test groups received either 2.0 mg/kg; 0.06 mg, 100 mg/kg; 0.3 mg or 20 mg/kg; 0.6 mg of Hf as a single dose in the vehicle (0.2 mL) 1 hr prior to anaesthesia. Sodium pentobarbitone (60 mg/mL) was diluted 1:20 with distilled water and injected (0.3 mL i.p.) 1 hr after the pre-treatment. Study (2): Thirty female, brown mice (mean weight = 30 g) were used in two groups (N = 15). A control group, administered methanol (10 μL; i.p.) and a test group administered Hf (20 mg/kg, 10 μL i.p.) both over a 4 day period. On the fifth day, sodium pentobarbitone (60 mg/mL), diluted 1:20 with distilled water was injected (0.3 mL; i.p.). Sleeping time was calculated

as the time elapsed from the loss of the righting reflex to it being regained.

Antipyrine metabolism in rats in vivo. Ap (2.5 mg) was administered orally to two groups of six rats. In group 1, the controls, the vehicle methanol was administered (100 μL, i.p.) while the test animals in group 2 received Hf in methanol (5 mg; 100 μL, i.p.). The animals were housed in metabolism cages and urine collected 0–8 hr and 8–24 hr. Concentrations of Ap, Np, 3-OH and 4-OH Ap were determined by HPLC [13]. On termination of the experiment, a blood sample (2.0 mL) was removed by cardiac puncture for determination of Hf and Hfm.

Hf and Hfm analysis

Analysis of perfusate. Perfusate concentrations of Hf and Hfm were determined by a selective, sensitive and reproducible HPLC method [14].

Analysis of bile. Hf and Hfm concentrations were determined by HPLC [14] in each hourly bile sample following enzyme hydrolysis of aliquots (10 μL) of bile. Sodium acetate buffer (0.1 M; 0.2 mL; pH 50) was added to each aliquot of bile in the presence or absence of sulphatase enzyme type H (2 mg/mL; 300 units/mg β-glucuronidase and 14–50 units/mg sulphtase; Sigma Chemical Co., Poole, U.K.) and incubated for 2 hr at 37°.

Analysis of liver tissue from perfusion experiments. Livers were homogenized with a teflon in glass homogenizer in phosphate buffer (0.067 M, pH 7.5) containing KCl (0.15 M). Concentrations of Hf and Hfm in the 25% homogenate were determined by HPLC [14].

Calculations

Pharmacokinetic variables for Ap were calculated using model independent formulae [15]. Biliary clearance was estimated by division of the amount of Hf eliminated in the bile over each 1 hr collection interval by the perfusate plasma concentration of Hf at the mid-point of that interval. K_m and V_{max} values for N-demethylation of Am and dealkylation of 7-Ec were obtained from double-reciprocal plots.

Statistical analysis

Comparison of the antipyrine pharmacokinetic variables ($t_{\frac{1}{2}}$, Cl, Vd), biliary clearance of Hf, righting reflex recovery time, K_m and V_{max} for the Am N-demethylation and 7-Ec O-dealkylation reactions were made by one-way analysis of variance. The significance of any changes in the concentration of antipyrine metabolites in rat urine and in activity of Am *N*-demethylase and 7-Ec *O*-deethylase on chronic dosage with Hf was determined by a non-paired Student's *t*-test, rejecting the null hypothesis for all tests if $P < 0.05$.

RESULTS

Effect of Hf on hepatic drug metabolism in vivo *and* in vitro

Increasing doses of Hf from (0.5–5.0 mg) did not alter the rate of elimination of antipyrine (Fig. 2) as demonstrated by no significant change in half-life and clearance values of Ap. No significant difference was observed in the apparant volume of distribution, with values equivalent to that of the volume of the perfusate reservoir (Table 1).

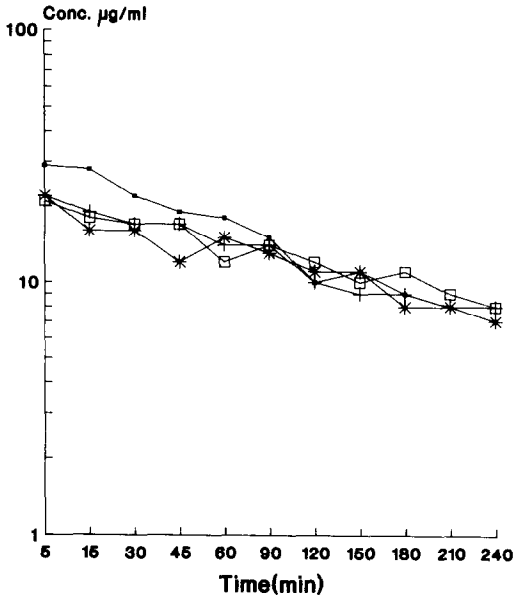

Fig. 2. Semilogarithmic plot of perfusate concentrations of antipyrine (μg/mL) following a single antipyrine dose (2.5 mg) as a control (filled square), and in the presence of 0.5 (cross), 2.5 (star) and 5.0 mg (open square) of halofantrine in the isolated perfused liver (IPRL) system (mean values, N = 5).

Table 1. Mean (SD) pharmacokinetic parameters for antipyrine (2.5 mg) after a single bolus dose (0.5, 2.5 and 5.0 mg) of halofantrine hydrochloride (Hf) into the perfusate reservoir (N = 5)

	$t_{\frac{1}{2}}$ (min)	Cl (mL/min)	Vd (mL)
Control	265 (50)	0.51 (0.11)	189 (14)
0.5 mgHf	275 (84)	0.48 (0.12)	177 (33)
2.5 mgHf	393 (82)	0.40 (0.10)	217 (18)
5.0 mgHf	338 (59)	0.40 (0.08)	188 (19)

$P > 0.05$ one-way ANOVA for all parameters. No significant effect of Hf.

Effects of Hf on hepatic metabolism carried out *in vitro* using microsomal incubations were determined using Am and 7-Ec as test substrates. The values of K_m and V_{max} of the enzymatic reactions showed no significant difference in the presence of increasing concentrations of Hf (0.01–0.1 mM) compared with the controls (Table 2). Incubations carried out with microsomes produced from rat livers chronically dosed with Hf on Am and 7-Ec substrates also produced no significant effect on the N-demethylation of Am or O-dealkylation of 7-Ec (Table 3). The concentration of Hf in each incubation, estimated by

Table 2. Mean (SD; N = 5) values of apparent K_m and V_{max} for substrates Am with units of (mM) and (nmol/min/mg protein), and Ec with units of (nM) and (pmol/min/mg protein), respectively after acute administration of halofantrine hydrochloride (Hf)

	Control		Hf (0.01mM)		Hf (0.05mM)		Hf (0.1mM)	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Ap	0.47 (0.20)	2.60 (0.19)	0.47 (0.22)	2.55 (0.21)	0.33 (0.050)	2.50 (0.06)	0.49 (0.11)	2.69 (0.16)
Ec	35.0 (18.6)	90.2 (31.7)	27.3 (5.4)	79.2 (11.7)	16.9 (0.80)	64.2 (1.74)	18.7 (0.90)	68.0 (2.00)

$P > 0.05$ one-way ANOVA, for all values. No significant effect of Hf.

Table 3. N-Demethylation of aminopyrine (2.5 mM; nmol/min/mg protein; mean (SD, N = 5) and O-dealkylation of ethoxycoumarin (150 nM; pmol/min/mg protein; mean (SD, N = 5) after chronic administration of halofantrine (20 mg/kg/day) compared with vehicle-dosed controls

	Control	Hf
Ap	1.93 (0.27)	2.29 (0.29)
Ec	112.0 (5.4)	128.0 (11.8)

$P > 0.05$ unpaired Student's t-test. No significant effect of Hf.

liquid scintillation spectrometry, ranged from 66 to 95% of that added initially.

Increasing acute oral doses of Hf (0.06, 0.3 and 0.6 mg) produced pentobarbitone induced sleeping time (min) values (mean ± SD) of 85 ± 12, 83 ± 21 and 81 ± 32, respectively, compared with a control value of 70 ± 17 (N = 12). These mean values were not significantly different.

Chronic dosage with Hf (20 mg/kg, i.p over 4 days) produced sleeping time values (mean ± SD) of 118 ± 71 min compared with control values of 100 ± 51, respectively (N = 15, $P > 0.05$). Following the oral administration of Ap, (2.5 mg) and Hf, (5.0 mg i.p.) total urinary recoveries (μg) of Ap and its three principal urinary metabolites, 3-OH, Np and 4-OH were determined. For the controls (N = 6) values were, (mean ± SD) of 142.6 ± 64, 729.4 ± 117.2, 76 ± 78.8 and 506.4 ± 131.1 (μg/mL), respectively. For rats receiving Hf the values were (N = 6) of 152.2 ± 43.6, 762 ± 120.7, 84.8 ± 75.7 and 476 ± 81.8 (μg), respectively. No difference between the control and test groups was observed at the 5% level of significance.

Perfusate disposition of halofantrine

The mean log Hf perfusate concentration/time curves for increasing Hf dose (0.5, 2.5 and 5.0 mg) are shown in Fig. 3. Hf and Hfm concentrations in the perfusate were determined at one hourly intervals, in bile and in the liver tissue homogenate. Hfm concentrations in the perfusate (4–31 ng/mL) rarely exceeded the minimum detectable concentration

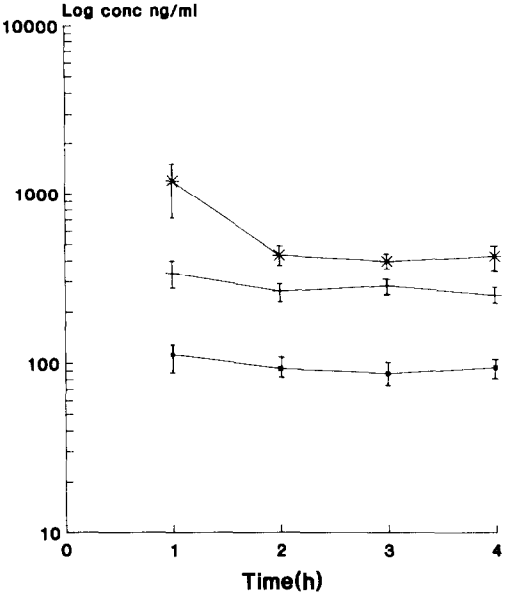

Fig. 3. Semilogarithmic of perfusate concentration of halofantrine (ng/mL) following the single dose administration of 0.5 (filled square), 2.5 (cross) and 5.0 mg (star) halofantrine hydrochloride to the IPRL system (mean ± SE, N = 5).

(1.0 ng/mL). Comparably small concentrations of Hfm were also found in the bile (80–200 ng/mL bile) and in the liver tissue (0.8–6.5 μg/mL homogenate). Cumulative biliary excretion and biliary clearance of Hf were determined (Figs 4 and 5, respectively). Only a small proportion of the dose was excreted in the bile (0.05–0.2%). Calculation of biliary clearance (Fig. 5) showed that there was a constant clearance of Hf into the bile over the dosage range during the 4 hr experimental period. At the conclusion of each experiment the amount of Hf present within the liver tissue was determined as a percentage of the administered dose (0.5, 2.5 mg and 5.0 mg) with a mean ± SD of 44.4 ± 16.4, 27.7 ± 6.7 and 28.2 ± 8.9%, respectively, so approximately 30% of the dose was present within the tissue. Assessment of Hf mass balance (mean ± SD) was performed at each dose level. For 0.5, 2.5 mg and 5.0 mg doses, respectively 46.2 ± 16, 29.5 ± 7.5 and 29.2 ± 9.0%

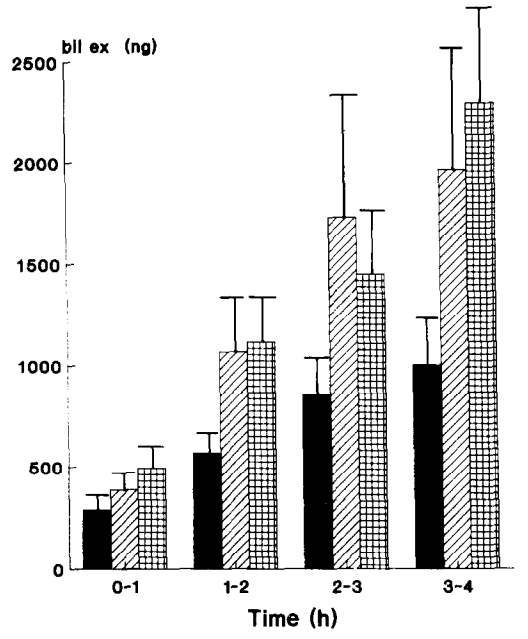

Fig. 4. Cumulative biliary excretion (ng) of halofantrine versus time (hr) after the administration of a single dose of 0.5 (filled bar), 2.5 (hatched bar) and 5.0 mg (cross hatched bar) halofantrine in the IPRL system (mean ± SE, N = 5).

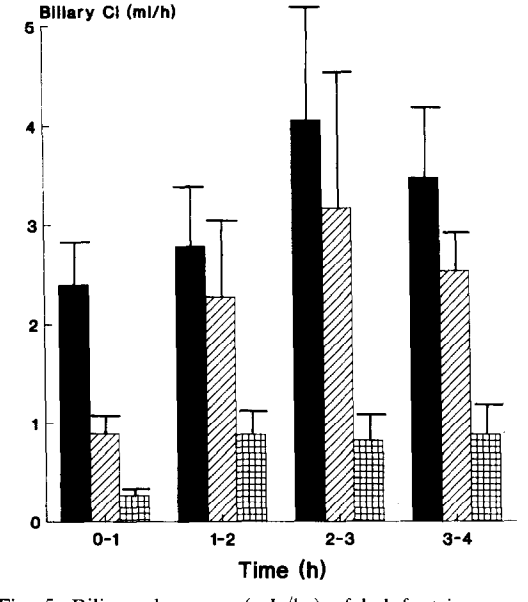

Fig. 5. Biliary clearance (mL/hr) of halofantrine versus time (hr) after the administration of a single dose of 0.5 (filled bar), 2.5 (hatched bar) and 5.0 mg (cross hatched bar) halofantrine hydrochloride to the IPRL system (mean ± SE, N = 5).

of the dose was recovered as Hf. The resulting concentrations (mean ± SD) of Hf present within each liver at the respective Hf doses were 0.41 ± 0.15, 1.29 ± 0.33 and 2.63 ± 0.83 μM.

Following oral administration of Ap, Hf and Hfm concentrations were determined in the plasma of the Hf dosed animals at 24 hr. Values (mean ± SD) were 347 ± 91 and 618 ± 120 ng/mL respectively.

DISCUSSION

We have demonstrated that Hf produces no significant inhibitory effects on hepatic drug oxidation. Ap disposition was determined in the IPRL and no differences in $t_{\frac{1}{2}}$, Cl and Vd values were found between the control and Hf dosed livers. This suggests that increasing dosage of Hf has no effect on the hepatic handling of Ap. Inspection of perfusate concentrations of Hf indicates a linear and proportional increase from 0.5–5.0 mg. In all the experiments, Hf appeared only to undergo hepatic uptake, with no further appreciable reduction thereafter. Analysis of the collected bile samples revealed that no unchanged Hf or Hfm was detected and only 0.2% of the dose was eliminated in the bile as conjugates with small amounts of Hfm detected in some samples. Biliary excretion data also suggest that this route of elimination is readily saturated as the dose of Hf is increased. Estimation of the amount of Hf within the liver demonstrated that ~ 30% of the dose was present as the parent drug in the liver at the termination of each experiment with only trace amounts of Hfm detected. Measurement of Hf concentrations within the liver allows the molar concentration of Hf retained in the liver to be determined (0.41–2.63 μM) for each dosage. The range of concentrations of Hf (0.93–9.3 μM) administered to the perfusate covered the IC_{50} values determined by Mihaly *et al.* [7] for primaquine (7.0 μM) and that reported by these authors for cimetidine (2.8 μM) using the same experimental model where these concentrations inhibited antipyrine clearance. That there was no inhibitory effect of Hf on Ap disposition is supported further by results of studies with microsomal preparations *in vitro* with Am and 7-EC drug substrates which indicate that Hf does not interfere with the enzymatic pathways involved with N-demethylation of Am and O-dealkylation of 7-Ec. Measurement of pentobarbitone induced sleeping time was also studied, in the presence of increasing Hf dose administered acutely and chronically. No change in pentobarbitone sleeping time was observed which indicates that Hf has no effect on the enzymatic system(s) involved in hepatic metabolism of the barbiturate. The possibility of selective inhibition by Hf *in vivo* of specific isoenzymes of cytochrome P450 was also investigated using antipyrine. It is generally accepted that the hepatic clearance of Ap its three principal metabolites is catalysed by different cytochrome P450 isoenzymes [16, 17]. In view of this, the effect of Hf on the production of the major metabolites of Ap; Np, 3-OH and 4-OH antipyrine was examined. No difference in urinary recovery of the individual metabolites of Ap was found.

We conclude, therefore, that Hf is not a potent or specific inhibitor of drug action *in vitro* or *in vivo*. This is in contrast to the findings of previous work carried out on other antimalarial agents. For example, Back *et al.* [18] demonstrated that the 8-aminoquinoline primaquine was a more potent

inhibitor of mixed function oxidase activity (MFO) than the 4-aminoquinoline chloroquine. Studies on primaquine enantiomers and its putative metabolites [7] also demonstrated inhibition of drug metabolism *in vitro*. Further investigations with amodiaquine, a 4-aminoquinoline, and mefloquine, a quinoline methanol also showed these antimalarials to be MFO system inhibitors [19, 20]. All of these inhibitors of drug metabolism contain a quinoline nucleus, a heterocyclic ring system which contains a nitrogen atom with lone pairs of electrons enabling an interaction with high spin cytochrome P450 and so inhibiting its action [1]. Changes in potency between the individual agents are dependent upon the structure and stereochemistry of each compound. Although Hf does not contain a quinoline nucleus, a free nitrogen atom is present in its side chain. The absence of inhibitory effects by Hf does suggest that this atom is either chemically inert or sterically hindered from any interaction with cytochrome P450.

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