The Stereoselective Distribution of Halofantrine Enantiomers Within Human, Dog, and Rat Plasma Lipoproteins

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Purpose. To study the *in vitro* distribution of the enantiomers of the antimalarial drug halofantrine in human, dog and rat plasma lipoprotein-fractions.

Methods. Plasma was spiked with racemic halofantrine (1000 ng/ml) and incubated for 1 h at 37°C. The fractions (high and low density lipoproteins, triglyceride-rich lipoproteins and lipoprotein deficient plasma) were separated using density gradient ultracentrifugation. Fractions were assayed for halofantrine enantiomer using stereospecific high performance liquid chromatography.

Results. The (−) enantiomer of halofantrine displayed higher affinity for the lipoprotein-deficient fraction than the (+) enantiomer in all three species. The (+) enantiomer was predominately located in the lipoprotein rich fractions of dog and human plasma (the (+):(−) ratio ranging from 1.2–9.6). In contrast, the (+):(−) ratio was consistently <1 in lipoprotein-deficient fractions. Dog displayed a large magnitude of stereoselectivity in halofantrine distribution to the plasma fractions tested. There were substantial interspecies differences in the pattern of distribution of halofantrine enantiomers within the different fractions. A significant positive relationship was observed between halofantrine uptake into lipoprotein-rich fractions and the percent of apolar core lipid in those fractions. There was also a strong negative correlation between total protein concentration and the enantiomeric ratio in the lipoprotein-deficient plasma fraction.

Conclusion. Distribution of halofantrine enantiomer to plasma lipoprotein-fractions is stereoselective and species specific. This differential binding of halofantrine enantiomers to lipoproteins may need to be considered in viewing pharmacokinetic and pharmacodynamic data involving the drug.

KEY WORDS: lipoprotein distribution; enantioselectivity; antimalarial.

INTRODUCTION

Halofantrine (HF) is an effective antimalarial agent in the treatment of infections caused by chloroquine-resistant strains of *P. falciparum* (1). The drug is highly lipophilic, and in association with this physicochemical characteristic HF possesses a high volume of distribution (Vd) in humans and different animal species (2–4). The drug also displays a marked increase in oral bioavailability after ingestion of a high fat-content meal (3,5), and shows a significant uptake into human plasma lipoproteins (6–8). Recently it was shown that in the dog, ingestion of a high fat meal caused post-prandial decreases of 14% and 22%, respectively, in the total body clearance (CL) and Vd of HF (9). This finding strongly suggests that lipoprotein binding of HF is an important determinant in the pharmacokinetics of the drug.

One feature that should be considered in interpreting pharmacokinetic data of HF is that the drug is chiral and administered as the racemate. Although there is evidence that HF possesses stereoselectivity in its pharmacokinetics in human (10) and rat (4), all of the available pharmacokinetic data related to the food-effect seen in humans and animals is nonstereoselective in nature. Given that the drug has the potential to elicit enantioselective cardiotoxicity (11), and that it is bound to plasma proteins to high extent (6), the data in hand related to the lipoprotein binding of the individual HF enantiomers is incomplete. To better understand the nature of the lipoprotein association of HF, we have studied and here report the relative distribution of HF enantiomers in plasma of rat, dog, and human. These are the three species that have been the focus of attention in stereoselective pharmacokinetic, food-effect, and lipoprotein association studies of HF.

METHODS

Chemicals

Racemic HF was a gift from SmithKline Beecham Pharmaceuticals (Worthing, UK). Sodium bromide, ethylenediaminetetraacetic acid (EDTA), imipramine (internal standard), and enzymatic assay kits for triglyceride and total protein were purchased from Sigma Chemical Company (St. Louis, MO). (+)-Di-O-acetyl-L-tartaric acid anhydride was purchased from Fluka (Ronkonkoma, NY). A free cholesterol assay kit was purchased from Boehringer Mannheim. All other chemicals were analytical grade or higher, and were purchased from Fisher Scientific (Fair Lawn, NJ).

Incubation of Plasma

Human plasma of six fasted (12–16 h) normolipidemic subjects (cholesterol and triglyceride levels between 100–200 mg/dl) was obtained from the Vancouver Red Cross (Vancouver, BC, Canada). Dog plasma was obtained from six fasted beagles and rat plasma was obtained from five fasted male Sprague-Dawley rats. Immediately after collection, 10 μl of 0.4 M EDTA (pH 7.1) was added to 1 ml of whole blood. Plasma was separated from blood cells by centrifugation of whole blood at room temperature for 10 minutes at 2000 g. A stock solution of racemic HF (200 μg/ml) was prepared in methanol. The addition of this methanolic solution to plasma did not modify the lipoprotein-lipid composition (8).

Each plasma sample (3 ml) was spiked with sufficient HF to provide for concentrations of 1000 ng/ml of the racemate. The samples were vortexed for 30 s and incubation was allowed to proceed for 60 min at 37°C prior to separation of lipoprotein fractions.

Lipoprotein Separation

The plasma was separated by step-gradient ultracentrifugation into its high (HDL) and low (LDL) density lipoprotein,

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its triglyceride-rich lipoprotein (TRL), which consists of very-low density lipoproteins and chylomicrons, and its lipoprotein deficient (LPDP) fractions as previously described (8). To confirm that HF recovery in the different density fractions is not a function of formulation density but of HF lipoprotein association, HF was incubated in LPDP controls (12).

Analytical Techniques

Determination of Total and Plasma Lipoprotein Triglyceride, Cholesterol, and Protein Concentrations

Total and plasma lipoprotein triglycerides, cholesterol (esterified and unesterified), and protein concentrations were determined as previously described (8,12). The external calibration curve for plasma and lipoprotein triglyceride was linear in a concentration range of 10–300 mg/dl ($r^2 > 0.95$). For cholesterol and total protein the standard curves were linear in a concentration range of 10–450 mg/dl ($r^2 > 0.96$) and 5–2000 mg/dl ($r^2 > 0.90$), respectively. Free cholesterol was determined using an enzymatic assay and was the calibration curve was linear in a concentration range of 1–100 mg/dl ($r^2 > 0.90$). Cholesteryl ester concentration was determined by calculating the difference between total and free cholesterol.

Stereospecific Assay of Halofantrine

Halofantrine enantiomers were assayed in 100 μl of each plasma fraction using a stereospecific assay method (13,14). Separate rat, dog and human plasma specific standard curves and quality control samples were generated for quantitation of HF enantiomer, for each of the fractions studied (HDL, LDL, TRL and LPDP). For each enantiomer standard curves had r^2 values of >0.99 in each of the fractions and runs, and the interday CV were less than 17% for each enantiomer. The validated lower limit of quantitation was 25 ng/ml of enantiomer in 100 μl of specimen (14).

Data and Statistical Analysis

Differences in the human plasma distribution of HF following incubation in human plasmas of varying lipid and protein concentrations were determined by a two-way analysis of variance (PCANOVA; Human Systems Dynamics). Critical differences were assessed by Neuman-Keuls posthoc tests. Single factor ANOVA was used to assess the difference of the enantiomeric ratios from unity. Regression analysis was undertaken to seek relationships between fraction composition and HF uptake. Correlation coefficients were determined for the relationship between apolar core lipid and HF uptake in the lipoprotein-rich fractions (LDL, HDL and TRL), and for total protein in the fractions versus HF uptake. Pearson's correlation coefficient was used to assess the strength of relationship in conjunction with level of significance. For all comparisons, differences were considered significant if $p < 0.05$. All data are expressed as mean ± SD.

Results

The plasma lipid profiles of each species noticeably differed in composition (Table 1). The cholesterol and triglyceride levels in the fractions were unique to each species, with little clear similarity in pattern between species. The dog possessed noticeably higher total protein levels in LPDP and HDL fractions compared to rat and human.

Table 1. Mean (±SD) Plasma Lipoprotein Cholesterol (Esterified & Unesterified), Triglyceride and Protein Concentrations (mg/dl) in Fractions of Normolipidemic Human, Dog, and Rat Plasma Samples[a]

Species	HDL	TRL	LDL	LPDP
Cholesterol (esterified & unesterified)				
Human	13.8 ± 1.0	26.3 ± 1.6	47.0 ± 3.4	—
Dog	48.3 ± 5.3	4.3 ± 1.3	53.7 ± 6.2	—
Rat	34.0 ± 2.2	1.5 ± 1.1	1.4 ± 1.3	—
Triglycerides				
Human	16.4 ± 0.6	65.1 ± 4.1	38.0 ± 2.6	—
Dog	7.2 ± 0.8	4.7 ± 1.3	11.1 ± 1.2	—
Rat	12.7 ± 1.0	8.2 ± 1.6	18.3 ± 1.6	—
Total Protein				
Human	64.8 ± 5.1	20.5 ± 2.5	56.0 ± 3.1	5350 ± 398
Dog	171.8 ± 32.6	1.8 ± 0.6	42.6 ± 6.5	12160 ± 716
Rat	33.5 ± 4.1	2.8 ± 1.3	55.2 ± 7.5	4411 ± 24

[a] Human and dog samples, n = 6; rat samples, n = 5. Abbreviations: TRL, triglyceride-rich lipoproteins (including chylomicrons and very low-density lipoproteins); LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein deficient plasma fraction (which includes α-1 glycoprotein and albumin).

With respect to HF distribution, there were numerous differences between species, and between fractions within species (Tables 2 & 3). In viewing the (−) enantiomer, all three mammalian species displayed a similar pattern, in that the bulk of the enantiomer present in plasma was recovered in the LPDP fraction (Table 2). There was more (−)-HF present in the lipoprotein-containing fractions in human plasma than in dog plasma (Table 2). In two of the rat HDL fractions, the concentrations of both HF enantiomers were below the lower limit of quantitation of the assay (25 ng/ml). In one each of dog HDL and TRL samples, the levels of (−)-HF were also below the lower limit of quantitation.

For (+)-HF, there was no consistent trend in disposition within plasma between the rat, dog and human (Table 2). Unlike the situation for (−)-HF, in dog and human plasma the (+) enantiomer was mostly present in the lipoprotein-rich fractions. However, in the rat both enantiomers were predominately recovered in the LPDP fraction. With respect to the sum of the enantiomers, (±)-HF showed an across-fraction pattern more similar to (−)-HF than (+)-HF in plasma within each of the three species (Table 2).

In the lipoprotein-rich fractions of each species, the enantiomeric (+):(−) ratio was significantly >1 (Table 3). Conversely, in the LPDP fractions the ratios were consistently and significantly <1. The dog displayed the highest degree of stereoselectivity as assessed by the (+):(−) ratios. In each of the lipoprotein-rich fractions, the enantiomeric ratio of (+):(−) HF between species consistently indicated that human < rat < dog (Table 3). The order was reversed for LPDP, with dog < rat < human (Table 3).

When the percent apolar lipid content in all lipoproteins samples and species was plotted versus HF enantiomer content, significant positive relationships (Fig. 1) were observed for the (+) enantiomer (ng/ml = 8.5 × %apolar lipid-3.6) and for (±)-HF(ng/ml = 11.1 × %apolar lipid + 38.8). On the other

Table 2. The Mean ± SD Plasma Lipoprotein and Lipoprotein-Deficient Distribution of Halofantrine (HF) Enantiomers [1000 ng/ml] Following Incubation for 60 Minutes at 37° C in Normolipidemic Human, Dog and Rat Plasma. Data Expressed as Percent of Initial HF Concentration Incubated. Between-Species and Between-Fraction Ranking Is Depicted in Ascending Order, Broken Underlines Indicate Significant Differences; Solid Underlines Indicate Nonsignificant Differences[a]

	HDL	TRL	LDL	LPDP	Between-fraction ranking ($p < 0.05$)
(+)-HF					
Human plasma	7.0 ± 1.0	12.7 ± 2.2	15.7 ± 5.1	11.9 ± 1.3	*HDL LPDP TRL LDL*
Dog plasma	17.4 ± 1.7	1.7 ± 0.5	19.6 ± 4.0	7.9 ± 0.5	*TRL LPDP HDL LDL*
Rat plasma	5.5 ± 0.5 (n = 3)	1.9 ± 0.3	10.3 ± 3.8	36.2 ± 6.5	*TRL HDL LDL LPDP*
Between-species ranking ($p < 0.05$)	*Rat human dog*	*Dog rat human*	*Rat human dog*	*Dog human rat*	
(−)-HF					
Human plasma	3.7 ± 0.8	8.8 ± 2.7	12.5 ± 5.1	26.9 ± 6.7	*HDL TRL LDL LPDP*
Dog plasma	2.0 ± 0.7 (n = 5)	0.8 ± 0.1 (n = 5)	2.3 ± 0.8	49.0 ± 4.2	*TRL HDL LDL LPDP*
Rat plasma	2.2 ± 0.1 (n = 3)	1.5 ± 0.3	6.8 ± 3.5	42.2 ± 9.8	*TRL HDL LDL LPDP*
Between-species ranking ($p < 0.05$)	*Dog rat human*	*Dog rat human*	*Dog rat human*	*Human rat dog*	
(±)-HF					
Human plasma	10.8 ± 1.6	21.4 ± 4.7	28.2 ± 10.1	38.3 ± 7.3	*HDL TRL LDL LPDP*
Dog plasma	19.3 ± 2.3	2.6 ± 0.7	21.9 ± 3.0	56.9 ± 4.3	*TRL HDL LDL LPDP*
Rat plasma	7.7 ± 0.6 (n = 3)	3.4 ± 0.5	17.1 ± 7.2	78.4 ± 15.9	*TRL HDL LDL LPDP*
Between-species ranking ($p < 0.05$)	*Rat human dog*	*Dog rat human*	*Rat dog human*	*Human dog rat*	

[a] n = 6 for human and dogs and n = 5 for rats except as indicated.

hand, there was no significant relationship observed between apolar lipid content and (−)-HF uptake (Fig. 1). The enantiomeric ratio was also dependent on the percentage of core apolar lipid. When the total protein content within the LPDP fraction in all samples and species was plotted (Fig. 2) versus (+)-HF:(−)-HF ratio, a significant inverse correlation was observed (ratio = 0.0025 × mg total protein + 1.06).

DISCUSSION

Binding of drugs to plasma proteins is a fundamental consideration in viewing plasma concentration versus effect relationships and in assessing the drug's pharmacokinetic properties. Using an indirect method, Cenni *et al*. estimated that (±)-HF is over 99% bound to plasma components (which includes albumin, α_1 acid glycoprotein and lipoproteins) (6). Furthermore, it has been shown that a significant proportion of this binding is due to association of (±)-HF with lipoproteins (6–8). The stereoselective plasma protein binding of HF enantiomers has not been studied to date. Although HF enantiomers do not differ in their *in vitro* intrinsic ability to eradicate *P. falciparum*, they do have different *in vivo* potencies, likely as a result of stereoselectivity in pharmacokinetic properties (15,16). In addition, one of the most serious adverse effects associated with HF use is prolongation of the electrocardiographic QT interval, which has the potential to lead to serious ventricular arrhythmias (5,17). Recently it was shown by X-ray crystallographic studies that, theoretically, HF enantiomers may have the potential to differ in this activity (11). Given this information, study of stereoselective aspects of HF pharmacokinetics and pharmacodynamics is warranted.

To our knowledge, this is the first report in which stereoselective disposition of a chiral xenobiotic into plasma lipoprotein-fractions has been examined in depth. The current results indicated that HF exhibits stereoselectivity in its distribution

Table 3. Enantiomeric Ratio (mean ± SD) of (+):(−)-Halofantrine Within Plasma Lipoprotein-Rich and Lipoprotein-Deficient Fractions Following the Incubation of (±)-Halofantrine (1000 ng/ml) for 60 Minutes at 37° C in Normolipidemic Human, Dog and Rat Plasma. Between-Species and Between-Fraction Ranking is Depicted in Ascending Order; Broken Underlines Indicate Significant Differences; Solid Underlines Indicate Nonsignificant Differences[a]

	HDL	TRL	LDL	LPDP	Between-fraction ranking ($p < 0.05$)
Human	2.01 ± 0.09	1.27 ± 0.01	1.18 ± 0.06	0.55 ± 0.08	*LPDP LDL TRL HDL*
Dog	9.55 ± 3.73 (n = 5)	1.94 ± 0.46 (n = 5)	9.08 ± 2.83	0.16 ± 0.02	*LPDP TRL LDL HDL*
Rat	2.48 ± 0.17 (n = 3)	1.32 ± 0.14	1.61 ± 0.27	0.87 ± 0.09	*LPDP TRL LDL HDL*
Between-species ranking ($p < 0.05$)	*Human rat dog*	*Human rat dog*	*Human rat dog*	*Dog rat human*	

[a] n = 6 for human and dogs and n = 5 for rats except as indicated.

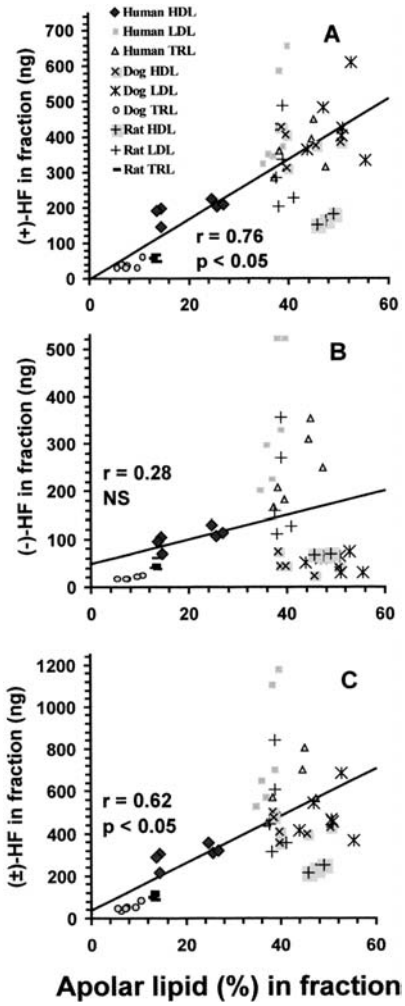

Fig. 1. Relationships between the percent of core apolar lipid in each of the lipoprotein samples (HDL, LDL and TRL) of rat, dog and human, and amount (ng) of A.) (+)-halofantrine, B.) (−)-halofantrine and C.) (±)-halofantrine, in each of those samples. Solid line represents best fit using linear regression analysis.

within and between the different lipoprotein fractions (Table 2). Notable interspecies differences were also observed in the distribution of HF enantiomers to the different fractions. It was a consistent observation that the (−) enantiomer resided to a greater extent in LPDP than did the (+) enantiomer (Tables 2 and 3). In terms of mass balance, for dog and human most of the (+) enantiomer resided within the confines of the lipoprotein-rich fractions, although the pattern of distribution of HF within these fractions was species-specific. The differences observed between species in HF distribution within plasma may be attributable to differences in lipoprotein and protein composition between each of the fractions (Table 1), and/or intrinsic differences in association of the HF enantiomers with those components of each species.

Both the CL and Vd of HF have been shown to be stereoselective in the rat after intravenous dosing (4). The stereoselective pharmacokinetics of HF have not been studied after iv dosing in humans or dogs, so in those species the actual CL and Vd of the enantiomers is not known. It is known, however, that HF possesses stereoselectivity in humans after oral doses (10),

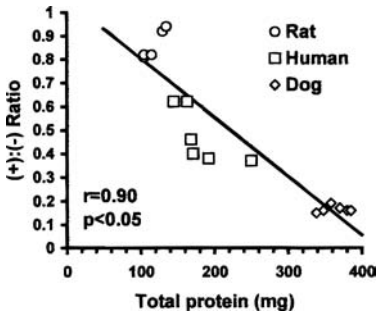

Fig. 2. The relationship between total protein (mg) and the enantiomeric ratio of halofantrine in the lipoprotein-deficient fractions of rat, dog, and human plasma. Solid line represents best fit using linear regression analysis.

and that the pattern of stereoselectivity is similar to that observed in the rat after oral doses. Humberstone *et al.* have shown that the CL of total (bound + unbound) (±)-HF decreases after ingestion of a high fat meal in the dog (9). This observation raises some interesting questions related to the outcome of this particular food versus drug interaction on the individual enantiomers, given the large degree of stereoselectivity seen in the lipoprotein binding in the dog (Tables 2 and 3) For example, the (+):(−) ratio of HF enantiomers in the HDL and LDL fractions in dog was greater than 9 (Table 3). Given that interspecies differences exist in the lipoprotein binding in each of these species, the influence of a fatty meal on the CL of HF enantiomers may not be uniform.

Similar to previous studies in which uptake of (±)-HF was related to the percent of apolar core lipid in the lipoprotein fractions (7,8), we found that the dispositions of (±)-HF and (+)-HF followed similar relationships when the data from the three species were grouped together (Fig. 1). There was a notable difference for (−)-HF, however, as its uptake into the different lipoprotein fractions was poorly related to core apolar lipoprotein lipid (Fig. 1). This enantioselectivity may be related to differences between the HF enantiomers in their relative binding within the lipoprotein-containing fractions and the LPDP fraction. Given that enantiomers share the same physicochemical properties in an achiral environment (18), the stereoselective differences in uptake within the lipoprotein-rich fractions are likely attributable to preferential binding of one of the enantiomers to components within the fractions. Apart from binding, it is also theoretically possible that solubility differences are rendered between the enantiomers within the microenvironment of the apolar lipid core of the lipoproteins, due to the presence of solubilized chiral modifiers.

The strong observed interspecies relationship (Fig. 2) between the total protein concentration in the LPDP fraction and the enantiomeric ratio was of interest, although difficult to explain. The LPDP fraction contains the major non-lipoprotein drug binding proteins of HF. With respect to the sum of mean albumin and α_1-acid glycoprotein concentrations (19), in descending order the species are ranked as rat (4.97 g/dl) > human (4.36 g/dL) > dog (3.0 g/dl). This is the same rank order as observed for the (+):(−)-HF ratio in the LPDP fraction (Fig. 2). Whether the enantiomeric ratio is truly related to total protein concentration, or whether it is a reflection of some other factor, however, remains open to question.

Recent studies have suggested that the binding of HF to lipoproteins may also alter its pharmacological effect. It has been shown that the IC_{50} of HF within an *in vitro* culture of *Plasmodium falciparum* was significantly decreased when incubated in the presence of 10% post-prandial serum (20). Halofantrine has a relatively low total body CL in human, dog and rat (2–4). Hence, it would be anticipated that changes in unbound fraction attributable to increased postprandial lipoprotein binding might have little impact on the *in vivo* situation, given that HF possesses a low hepatic extraction ratio. As a result, any change in binding should be offset by compensatory changes in CL which results in no net change in unbound HF concentration in plasma, even though total (bound + unbound) HF concentration may be significantly increased. Taken in context with the stereoselective binding of enantiomers to lipoproteins, it might be anticipated that the total (bound + unbound) concentrations of (+) enantiomer would be affected to a greater extent than the (−) enantiomer due to postprandial increases in lipoprotein content in plasma. This might be surmised based on the higher binding of the (+) enantiomer to lipoprotein-containing fractions (Table 2), and to the apparent dependence of apolar core lipids in that binding (Fig. 1).

In conclusion, the binding properties of HF are stereoselective in different fractions of human, dog and rat plasma. The results indicated that in comparison to its antipode, the (+) enantiomer is preferentially associated with lipoprotein-rich fractions of plasma, and the (−)-enantiomer is primarily associated with lipoprotein-deficient fractions. Careful evaluation and interpretation may be necessitated in viewing measurements of total (bound + unbound) plasma concentrations of HF.

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