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A simplified liquid chromatography assay for the quantitation of halofantrine and desbutylhalofantrine in plasma and identification of a degradation product of desbutylhalofantrine formed under alkaline conditions

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Abstract: The development of a new and simplified, validated LC assay for the quantitation of halofantrine and desbutylhalofantrine in plasma is described. The methodology employs an inexpensive, rapid and simple liquid–liquid extraction procedure in combination with previously reported chromatographic conditions. The method has been employed to study aspects of the pharmacokinetics of orally administered halofantrine in beagle dogs and some preliminary data are presented. During development of the extraction procedure, degradation of desbutylhalofantrine was observed under non-acidic conditions in the extraction solvent (*tert*-butyl methyl ether) and we also report the structural elucidation of the breakdown product and the conditions required to avoid this degradation.

Keywords: *Halofantrine; plasma assay; stability; LC.*

Introduction

The world-wide resurgence of malaria is of major therapeutic concern and new and effective treatment modalities are urgently required in light of the widespread resistance to chloroquine of the most dangerous malaria parasite, *Plasmodium falciparum*, and the emerging resistance to other widely available anti-malarial drugs [1–3].

Halofantrine (Hf), a 9-phenanthrene-methanol derivative, is well tolerated after oral administration and has confirmed efficacy against *Plasmodium falciparum*, including multi-drug resistant strains [1, 3]. Consequently, there is considerable and growing clinical interest in the use of Hf as an alternative treatment for malarial infections. Halofantrine is poorly and variably absorbed after oral administration [4] and consequently therapeutic drug monitoring is often employed to evaluate therapy. As the desbutyl metabolite of Hf (*N*-desbutylhalofantrine, Hfm) is also biologically active [1], the pharmacokinetic study of Hf requires measurement of the plasma concentrations of parent compound and the metabolite.

LC assays for the determination of Hf and Hfm from plasma have been described [5–8]. However, the methods reported by Milton *et al.* [5] and Gawienowski *et al.* [6] involve long analysis times (22 min) and employ complicated and expensive solid phase extraction procedures. Keeratithakul *et al.* [7] reported a more rapid chromatographic method but drug extraction utilized the solid phase procedure reported by Milton *et al.* [5]. This paper describes the validation of a new and simplified method for the determination of Hf and Hfm in plasma. The methodology employs an inexpensive, rapid and simple liquid–liquid extraction procedure in combination with previously reported chromatographic conditions [7]. The method has been employed to study aspects of the pharmacokinetics of Hf in beagle dogs and some preliminary data are

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presented. During development of the extraction procedure, degradation of Hfm under non-acidic conditions in the extraction solvent (*tert*-butyl methyl ether) was observed, and we also report the structural elucidation of the breakdown product and the conditions required to avoid this degradation.

Materials and Methods

Chemicals

Halofantrine HCl, desbutylhalofantrine HCl and the internal standard (IS) 2,4-Dichloro-6-trifluoromethyl-9-{1-(2-(dibutylamino)ethyl]}-phenanthrenemethanol HCl were obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). Halfan® tablets containing 250 mg Halofantrine HCl were obtained from SmithKline Beecham (Hertfordshire, UK). Acetonitrile (Mallinckrodt, KY, USA) and *tert*-butyl methyl ether (Fluka, Switzerland) were LC grade. Analytical grade hydrochloric acid and glacial acetic acid were purchased from Ajax Chemicals (Sydney, Australia). Electrophoresis grade sodium dodecyl sulphate (SDS) was obtained from Eastman Kodak (NY, USA). All other chemicals were of analytical reagent grade. Water was obtained from a Milli-Q (Millipore, Milford, MA, USA) water purification system.

Chromatography

Chromatography was performed according to the method of Keeratithakul *et al.* [7]. The LC system consisted of a Waters 590 programmable pump, a Waters 712 WISP injector, a Waters 486 absorbance detector (Millipore, Milford, MA, USA) set at 257 nm, an Ultrasphere C8 bonded-phase column (5-μm particle size, 25 cm × 4.6 mm i.d., Beckman Instruments, CA, USA) and a Newguard RP-8 cartridge guard column (Aquapore 7 μm, 3.2 × 15 mm, Applied Biosystems, CA, USA). Data were analysed on a Shimadzu C-R6A integrator (Shimadzu Corp., Japan). The mobile phase consisted of 75:25 (v/v) acetonitrile–water with 0.2% (w/v) SDS and 0.2% (v/v) glacial acetic acid. The flow rate was 1.5 ml min^{-1} and all separations were performed at room temperature.

Extraction procedure

A 1 ml aliquot of acetonitrile and 200 μl of IS spike (2 μg ml^{-1} in acetonitrile) were added to 0.5 ml of plasma in a 12 ml polypropylene centrifuge tube, the sample was vortexed for 2 min to precipitate plasma proteins, centrifuged, and then an 8 ml aliquot of *tert*-butyl methyl ether (TBME) was added. The contents were mixed by vortexing for 2 min and then centrifuged at 700*g* for 5 min. An 8-ml portion of the upper TBME layer was carefully removed using a glass pipette, placed in a polypropylene centrifuge tube containing 100 μl of 0.005 M HCl (in acetonitrile) and the contents evaporated to dryness under a stream of high purity N_2 at 35°C using a N-EVAP 112 evaporator (Organomation, MA, USA). The residue was reconstituted with 200 μl of acetonitrile and 25 μl was injected on to the LC column.

Standards and calibration

Stock solutions of Hf, Hfm and the IS were prepared at a concentration of 100 μg ml^{-1} (concentrations of Hf, Hfm and IS are reported as mass of base/ml) by dissolving the corresponding HCl salt in acetonitrile and the solutions were stored in glass at −20°C. Standard solutions were prepared in acetonitrile on each day of analysis at concentrations in the range of 0.05–20 μg ml^{-1} for Hf and Hfm, and at 2 μg ml^{-1} for the IS. Standard curves were prepared by plotting peak height ratios against concentration following extraction and analysis of spiked plasma samples as described. Unknown sample concentrations were calculated from the standard equation $y = mx + b$, as determined by linear regression of the standard curve, and linearity confirmed by calculating the correlation statistics of the regression line.

Assay recovery, precision and accuracy

Recovery was calculated by comparison of the peak heights of Hf, Hfm and the IS recovered from spiked plasma samples with the peak heights of the injected standard solutions. Intra-assay precision and the accuracy of the assay (expressed as ((mean observed concentration)/(expected concentration) × 100) were determined by replicate analyses ($n = 5$) of spiked plasma samples at three different concentrations (20, 500, 2000 ng ml^{-1}), and inter-assay precision determined by analysis of spiked plasma samples ($n = 5$) at these concentrations on three different days of analysis.

In vivo *application*

A two-way crossover study (7 day washout period) was conducted in a healthy male beagle dog (2 yr, 14.5 kg) after oral administration of a 250-mg Halfan® tablet in either the fed of fasted state. The dog was fasted for 12 h prior to each study day and water was available *ad libitum*. When the tablet was administered in the fed state, the fasted dog was given 600 g of standard canned dog food (typical composition of 2.5% fat and 7.5% protein) approximately 30 min prior to drug administration. Blood samples (2.5 ml) were obtained from the cephalic vein, either via an indwelling catheter or by individual venipuncture, at pre-dose and at 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 32, 48 and 72 h after drug administration. The blood samples were collected in sterile tubes containing 4.5 mg dipotassium EDTA. The plasma was separated by centrifugation at 1000*g* for 10 min and stored at −70°C until analysis. The peak plasma concentration (C_{max}) of Hf and Hfm, and the time of occurrence (T_{max}), were noted directly from the plasma profiles and the area under the curve ($AUC^{(0\rightarrow t)}$) of the plasma concentration vs time profiles were calculated using the linear trapezoidal rule.

Degradation of Hfm and structural characterization

During development of the TBME-based procedure, it became apparent that up to 10% of Hfm degraded during the extraction. The retention time of the Hfm degradation product was typically 6.4 min compared with 5.2 min for authentic Hfm. As TBME was an efficient solvent for extracting Hf and Hfm from plasma, the basis for the degradation was investigated with a view towards identifying conditions which prevented degradation of Hfm.

A sample of the Hfm degradation product was isolated from a solution of Hfm prepared in TBME and characterized by mass spectrometry (MS) using a Finnigan MAT 95 high resolution spectrometer. For MS analysis, compounds were introduced into the mass spectrometer using a direct probe heated from 100 to 350°C over 2 min and ionized by ammonia chloride ionization. Typical conditions were a source temperature of 160°C; electron energy of 70 eV; emission current of 150 mA; ion source pressure of 10^{-4} millitorr (convection gauge adjacent to the ion source); and resolution of 1 in 1000. Conditions for accurate mass spectra were identical except that a resolution of 1 in 5000 was used. Linked scans were performed with B/E in constant mode using He as the collision gas. The ammonia chloride mass spectra of Hfm ($C_{22}H_{22}F_3N_1O_1Cl_2$) gave an intense ion at *m/z* 444 corresponding to $[M + H]^+$. The degradation product mass spectra showed an intense ion at *m/z* 456, 12 atomic mass units (amu) higher than Hfm. Accurate mass spectra of this degradation product gave a pseudomolecular ion of 456.105 corresponding to $C_{23}H_{22}F_3N_1O_1Cl_2 + H$ (calculated mass 456.1108).

A further sample (3 mg) of the Hfm degradation product was produced by incubating Hfm in an alkaline methanolic solution (exposed to day-light) and chromatographic and MS data confirmed that the product was identical to the degradation product isolated from the TBME solution. The sample was dried under a stream of N_2 gas, reconstituted in $[^2H_6]$ dimethyl sulphoxide and ^1H NMR spectra were acquired at 300.13 MHz and 300 K on a Bruker AM300 spectrometer. Chemical shift values (δ) were accurate to 0.01 ppm and are reported relative to the internal tetramethyl silane (TMS) resonance at δ = 0.00. One dimensional (1D) spectra were recorded in 32k data points and the data were multiplied by an exponential window function and zero-filled to 64k prior to processing. The two dimensional spectra (2D) were acquired in phase sensitive mode using time proportional phase incrementation (TPPI) in both dimensions. For the double quantum filtered correlated spectroscopy (DQFCOSY) experiments, 128 t_1 increments were recorded with a sweep width of 3000 Hz and 2k data points. Phase sensitive NOESY spectra were acquired with a mixing time of 200 ms and consisted of 256 t_1 increments and were recorded with a sweep width of 3000 Hz and 2k data points. Prior to processing the 2D spectra, a cosine squared window function was applied and the data were zero-filled to 2k × 1k. The spectral assignments, made on the basis of chemical shift values and the splitting patterns of the peaks in the 1D spectra, are summarized in Table 1. The assignments were verified on the basis of through-bond and through-space connectivities observed in the 2D-DQFCOSY and NOESY spectra, respectively.

Table 1
Chemical shift and signal assignment of the 1D-NMR spectra of desbutylhalofantrine (Hfm) and the Hfm degradation product. Refer to Fig. 1 for numbering sequence used in peak assignments

Desbutylhalofantrine (Hfm)	Hfm degradation product
9.40 (s, 1; 5-CH)	9.38 (s, 1; 5-CH)
9.25 (s, 1; 4-CH)	9.24 (s, 1; 4-CH)
8.66 (s, 1; NH-)	Not present
8.57 (d, 1; 8-CH)	8.50 (d, 1; 8-CH)
8.48 (s, 1; 10-CH)	8.37 (s, 1; 10-CH)
8.06 (s, 1; 2-CH)	8.06 (s, 1; 2-CH)
8.05 (d, 1; 7-CH)	8.05 (d, 1; 7-CH)
6.11 (s, 1; hydroxyl)	Not present
5.59 (m, 1; 12-CH)	5.38 (m, 1; 12-CH)
Not present	4.72 (d, 1; O-CH_a-N)
Not present	4.53 (d, 1; O-CH_b-N)
3.10 (m, 1; 15a-CH_2)	3.11 (m, 2; 15a,b-CH_2)
3.22 (m, 1; 15b-CH_2)	
2.89 (m, 2; 16-CH_2)	2.72 (m, 2; 16-CH_2)
2.25 (m, 1; 14a-CH_2)	1.98 (m, 1; 14a-CH_2)
2.10 (m, 1; 14b-CH_2)	1.81 (m, 1; 14b-CH_2)
1.57 (m, 2; 17-CH_2)	1.48 (m, 2; 17-CH_2)
1.32 (m, 2; 18-CH_2)	1.36 (m, 2; 18-CH_2)
0.88 (m, 3; 19-CH_3)	0.93 (m, 3; 19-CH_3)

Results and Discussion

Recently reported methods for the determination of Hf and Hfm (Fig. 1) in biological fluids have generally employed solid phase extraction procedures, followed by a liquid–liquid back extraction step to prepare samples for LC analysis [5–7]. The need for a rapid and simple assay for Hf and Hfm has been addressed by Mberu *et al.* [8] who reported a simplified although less sensitive technique. All these methods have required the use of silanized glassware. We have developed a simplified and sensitive method for the determination of Hf and Hfm in plasma, which utilizes polypropylene tubes, involves a single step liquid–liquid extraction using *tert*-butyl methyl ether (TBME), and is both rapid and inexpensive.

The development of an efficient extraction procedure for Hf using TBME was relatively straight forward, whereas initial attempts to construct a linear standard curve for Hfm with acceptable precision were not successful due to variable peak height ratios from replicate analyses. A lower than expected peak height for Hfm corresponded with appearance of a new peak eluting approximately 1.2 min after Hfm. Analysis of each stage of the extraction procedure indicated that formation of the degradation product was associated with the presence of TBME. As formation of the Hfm degradation product was facile under alkaline conditions (and did not occur for the parent Hf), the conditions which promoted its formation were determined and the structure of the degradation product was elucidated as its formation has not been previously reported.

Degradation of Hfm in TBME

The degradation of Hfm in TBME only occurred under neutral/alkaline conditions, and an authentic sample was prepared by incubating Hfm in an alkaline methanolic solution exposed to day light. The chromatographic and MS characteristics of the prepared degradation product were identical to the product formed during extraction with TBME under neutral/alkaline conditions. This observation suggested that small quantities of methanol which may be present in LC grade TBME were most likely involved in the formation of the Hfm degradation product.

From the MS studies, the mass of the Hfm degradation product was 12 amu larger than the parent Hfm. Furthermore, the mass spectrum of the product formed after incubation of Hfm with alkaline ethanol gave an intense ion at *m/z* 470 (26 amu higher than the parent Hfm). This suggested that methanol or ethanol could react with Hfm with the concurrent loss of H_2O and H_2 where the loss of H_2 could occur through formation of either a double bond or a ring system. When Hfm was incubated with deuterated methanol ($^2H_3CO^2H$) the *m/z* ion of the degradation product shifted by only 14 amu corresponding to ^2H_2CO. This result was consistent with formation of a ring system rather than formation of a double bond. Using MS/MS analysis (linked scans, B/E mode) of the $[M + H]^+$ ion from the degradation product, it was evident that both the aromatic ring system and the *n*-butyl side chain attached to the nitrogen atom remained intact. However, the structure of the degradation product between the aromatic ring system and the nitrogen remained unclear and it could not be unequivocally established if a ring system or a double bond was present in the degradation product.

NMR analysis of the Hfm degradation product indicated that the aromatic ring system and *n*-butyl side chain were intact, consistent with the MS/MS data. The chemical shift, coupling constants and the peak multiplicities indicated that the protons associated with the carbon atoms in positions 12, 14 and 15 of the parent Hfm were present in that portion of the molecule of the degradation product (Table 1).

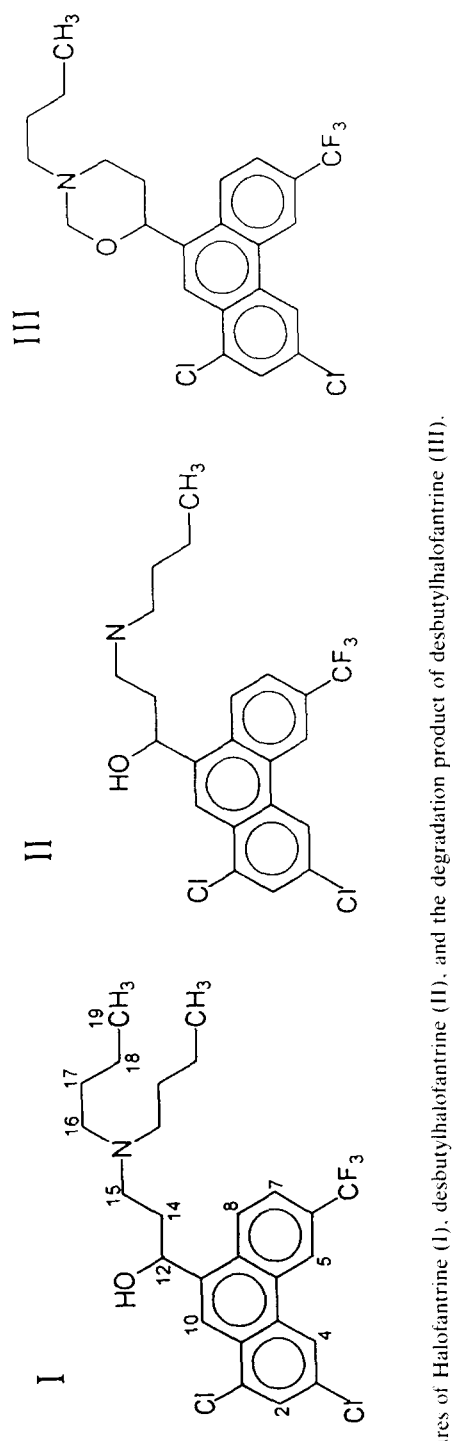

Figure 1
Chemical structures of Halofantrine (I), desbutylhalofantrine (II), and the degradation product of desbutylhalofantrine (III).

The only major difference in the NMR spectra of the degradation product and Hfm was the appearance of two peaks in the spectrum at 4.53 and 4.72 ppm and the loss of the peaks corresponding to the hydroxyl proton on carbon atom 12 and the proton present on the nitrogen atom. The integration of each peak at 4.53 and 4.72 ppm represented a single proton, and the doublet of doublets splitting pattern suggested that the two protons were non-equivalent and isolated (in terms of spin–spin coupling) from other protons in the molecule. When these observations were assessed in the context of the MS analysis (which indicated an increase of 12 amu between the aromatic ring system and the *n*-butyl side chain), it was apparent that the degradation product contained a 1,3 saturated oxazine ring structure as depicted in Fig. 1.

From a mechanistic standpoint, (i) the reaction required the presence of at least one proton on the side chain nitrogen atom as degradation was not observed with Hf, (ii) acidification of the solvent prevented the reaction, and (iii) the reaction appeared to require the presence of light. The most simple explanation for formation of the saturated oxazine ring is via addition of formaldehyde to the secondary nitrogen of Hfm forming a Schiff base which then collapses to the product. The addition of formaldehyde (as a contaminant of methanol or *tert*-butyl methyl ether) to 3-aminopropanol is a facile reaction [9]. Further studies are in progress to address the apparent requirement for light and whether the susceptibility of anti-malarials to photolytic process [10] plays a role in the formation of the oxazine-based degradation product.

In terms of assay development, the knowledge that acidic conditions prevented the reaction (by protonating the amine) led to the rational addition of 100 μl of 0.005 M HCl to the TBME plasma extract to limit degradation of Hfm and thereby improve the reproducibility and precision of the procedure to an acceptable level.

A small peak (1–2% of Hfm peak height) with a similar retention time to the Hfm degradation product is occasionally observed in chromatograms of extracted plasma. In the absence of an alternative method of analysis, which does not potentially introduce the Hfm degradation product, it is not possible to determine whether formation of the Hfm degradation product could actually occur *in vivo*. However, considering the proposed reaction pathway, this particular mechanism is unlikely to occur *in vivo*.

Assay characteristics

Figure 2 presents representative chromatograms of spiked and control plasma samples which had been extracted using the TBME procedure. Baseline resolution of Hf and Hfm within a 12 min run time was obtained, the retention times for Hfm, Hf and the IS were 5.2, 7.8 and 10.4 min, respectively, and the lowest level of quantitation was 10 ng ml^{-1} for

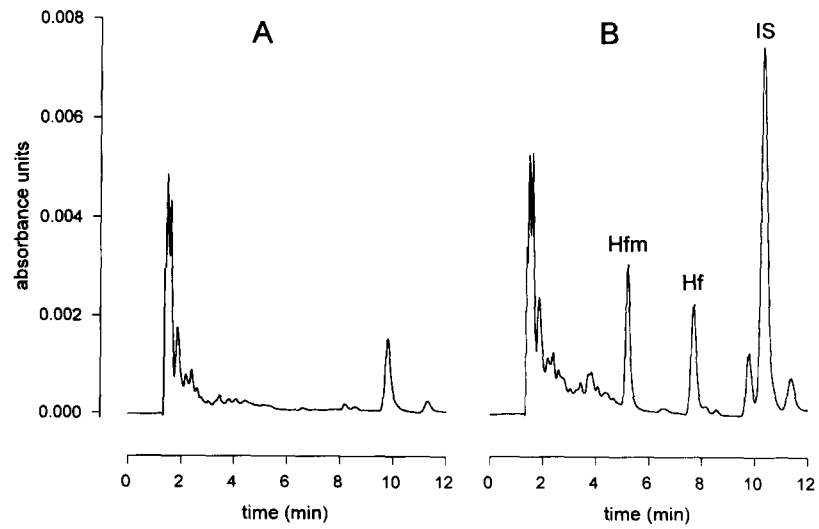

Figure 2
Representative chromatograms of extracted blank plasma (panel A), and an extracted plasma sample spiked with Hf and Hfm (corresponding to 200 ng ml^{-1} in plasma) and IS (panel B).

Table 2
The recovery, inter-assay and intra-assay precision, and accuracy of the assay procedure for Halofantrine (Hf) and Desbutylhalofantrine (Hfm) from spiked plasma samples

Compound	Conc. (ng ml^{-1})	% Recovery (mean ± SD)	Inter-assay precision (RSD, %)	Intra-assay precision (RSD, %)	Accuracy (%)
Hf	20	96.5 ± 9.1	2.2	4.5	101.9
	500	88.0 ± 2.6	4.7	0.7	99.9
	2000	90.8 ± 2.5	3.8	1.1	99.8
Hfm	20	91.4 ± 8.8	1.1	5.6	108.9
	500	87.7 ± 3.2	6.4	0.5	103.9
	2000	89.8 ± 5.1	7.8	4.5	100.2

both Hf and Hfm. Standard curves were linear in the spiked plasma concentration range 0–8000 ng ml^{-1} ($r^2 > 0.99$) and the recovery, precision and accuracy data are presented in Table 2. The average recoveries for Hf and Hfm were approximately 90% across the concentration range and the mean (±SD, $n = 20$) recovery of the IS was 82 ± 2%. The intra- and inter-assay precision and accuracy were highly satisfactory as the relative standard deviation (RSD) of the procedure was less than 8% for Hfm and less than 5% for Hf at each concentration studied.

The extraction and analytical procedure can be readily adapted to quantitate Hfm and Hf in whole blood. This is achieved by initially lysing the red blood cells (freezing then thawing the whole blood sample), adding 0.5 ml of water to a 0.5-ml aliquot, and then vortexing and sonicating the sample. The extraction efficiencies for Hfm, Hf and IS from spiked whole blood were similar to those obtained from spiked plasma (unpublished data).

The assay was employed to study aspects of the pharmacokinetics of Hf and Hfm in beagle dogs and a full report of the absolute bioavailability and pharmacokinetic data will be subsequently published. The following representative plasma profiles are presented to demonstrate the utility of the assay. Figure 3 depicts the plasma concentration vs time profiles for Hf and Hfm in a single dog after administration of 250 mg Hf.HCl in either the fed or fasted state. Administration of Hf with food increased the C_{max} of Hf 19-fold from 317 ng ml^{-1} (fasted) to 6014 ng ml^{-1}, and an concomitant 2.5-fold increase in the C_{max} for Hfm from 33 ng ml^{-1} (fasted) to 81 ng ml^{-1} (fed) was observed. There was an 11-fold increase in the plasma $AUC^{0\rightarrow 72h}$ of Hf from 4785 ng h ml^{-1} (fasted) to 53 980 ng h ml^{-1} (fed) and food also increased the plasma $AUC^{0\rightarrow 72h}$ for Hfm from 1934 ng h ml^{-1} (fasted) to 4983 ng h ml^{-1} (fed). The effect of food on the bioavailability of Hf in the single dog described in Fig. 3 was typical of the effect observed across the full study (unpublished data). The magnitude of the "food-effect" is consistent with human data where a 4-fold increase in the plasma AUC of Hf was observed after co-administration with food [4].

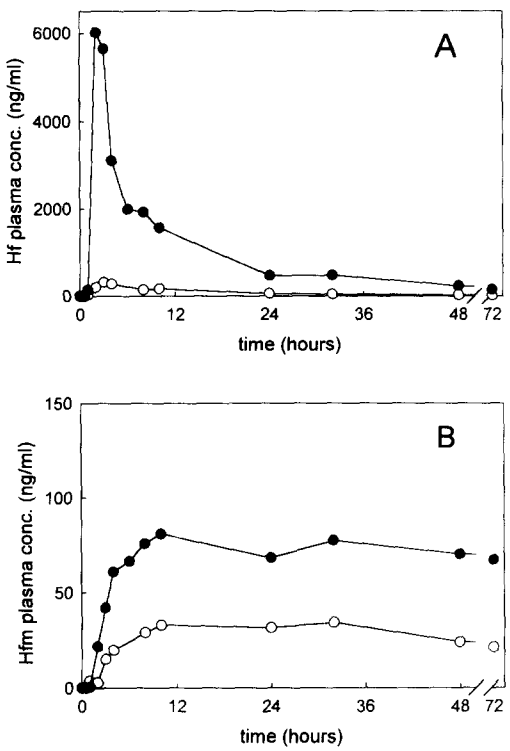

Figure 3
Plasma concentration time profile of Hf (Panel A) and Hfm (Panel B) after oral administration of 250 mg Halofantrine HCl to a male beagle dog in either the fed (●—●) or fasted state (○—○).

In summary, a simplified and inexpensive assay procedure has been rationally developed for the extraction of Hf and Hfm from plasma. The utility of the assay has been demonstrated in a pre-clinical bioavailability study and the

assay would have application to therapeutic drug monitoring and study of the pharmacokinetics of Hf. The structure of the degradation product of the desbutyl metabolite of Hf formed in alkaline alcoholic solution has been defined and the conditions required to avoid its formation have been identified.

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