Journal of Pharmaceutical & Biomedical Analysis
Vol. 10, No. 2/3, pp. 245–250, 1992
Printed in Great Britain

0731–7085/92 $5.00 + 0.00
Pergamon Press Ltd

Short Communication

The determination of the enantiomers of halofantrine and monodesbutylhalofantrine in plasma and whole blood using sequential achiral/chiral high-performance liquid chromatography

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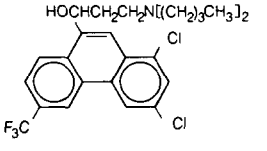
Keywords: *Multidimensional chromatography; reversed-phase liquid chromatography; chiral chromatography; pharmacokinetics; halofantrine.*

Introduction

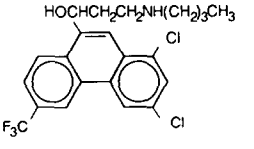
Halofantrine hydrochloride, 1,3-dichloro-6-trifluoromethyl-9-[1-hydroxy-3-(di-*n*-butylaminopropyl) phenanthrene] hydrochloride (HF), is an antimalarial drug active against chloroquine-resistant *Plasmodium falciparum*. It is mainly metabolized to its monodesbutylated derivative (HFM) which is also pharmacologically active as an antimalarial agent. Both HF and HFM possess asymmetric carbons (Fig. 1) and exist as enantiomeric pairs. The parent compound, HF, is administered as a racemic (50:50) mixture of the enantiomers.

Previous studies have shown that the pharmacokinetics and disposition of the chiral antimalarial agents mefloquine [1] and chloroquine [2] are stereospecific. However, a pharmacokinetic study of HF [3] did not examine the disposition of the separate HF of HFM enantiomers primarily because of the lack of an enantioselective assay. The previously reported HPLC assays of HF [3–5] were not designed to stereochemically resolve the HF and HFM enantiomers.

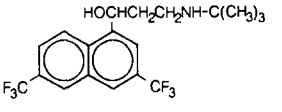
There have been two approaches to the development of stereoselective HPLC assays for the determination of chiral antimalarial agents in biological fluids. One method utilized coupled achiral/chiral HPLC columns (for



1: Halofantrine



2: Monodesbutylated Metabolite of Halofantrine



3: Internal Standard (ZN 31846)

Figure 1
Molecular structures of HF, HFM and IS.

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mefloquine [1]) and the other sequential achiral/chiral chromatography (for chloroquine [2]).

In the coupled achiral/chiral approach, mefloquine was initially separated from interfering substances in the biological matrix and quantified on an achiral column containing a cyano bonded stationary phase [2]. The eluent containing the mefloquine was selectively transferred to a silica pre-column through an on-line switching valve. The pre-column was used to concentrate the mefloquine which was then backflushed on to the second HPLC column which contained a (*S*)-naphthylurea chiral stationary phase. The mefloquine enantiomers were stereochemically resolved on the chiral stationary phase (CSP) and the enantiomeric ratio was determined.

In the sequential achiral/chiral chromatographic system used for the study of the pharmacokinetics of the enantiomers of chloroquine and its desethyl metabolite [2], chloroquine and desethylchloroquine were separated from each other and from interfering substances in the biological matrix and were quantified on an achiral column containing a silica bonded stationary phase. The eluents containing chloroquine and desethylchloroquine were separately collected and the fractions were evaporated to dryness, reconstituted and injected on to the chiral stationary phase which contained immobilized α_1-acid-glycoprotein. The enantiomeric ratios of the chloroquine stereoisomers and the desethylchloroquine stereoisomers were determined on the CSP.

This paper reports the development, validation and initial application of a sequential achiral/chiral HPLC method for the determination of the enantiomers of HF and HFM in plasma and whole blood. The assay is based upon the stereochemical resolution of the HF and HFM enantiomers on a protein-based CSP prepared by immobilization of ovomucoid [6]. This CSP has been previously used for the stereochemical separation of basic compounds, such as chlorpheniramine and chlorphenesin, as well as acidic solutes like ibuprofen and flurbiprofen.

The ovomucoid chiral stationary phase (OVM-CSP) stereochemically resolved the enantiomers of HF and HFM. However, this separation could not be directly applied to samples extracted from plasma and whole blood because of chromatographic interference from endogenous compounds. One of the key requirements for a coupled column system is to find a compatible mobile phase for both systems. It was not possible to find an achiral stationary phase which would resolve HF and HFM from each other and from the matrix interference using a mobile phase which was suitable for the OVM-CSP. For this reason, a sequential achiral/chiral system was developed.

In this sequential system, an achiral RP-2 stationary phase was first used to separate HF and HFM from each other and from other interfering compounds. The total amounts of HF and HFM were determined on the achiral system. The eluent fractions containing HF and HFM were collected separately, evaporated and injected into the OVM-CSP after reconstitution in methanol. The enantiomeric ratios of HF and HFM were then determined. This system has been validated and used in the determination of serum and whole blood concentrations of HF and HFM after the administration of a single dose of racemic HF.

Experimental

Chemicals

Racemic halofantrine (rac-HF) and its racemic desbutylated metabolite (rac-HFM) were gifts from SK&F companies (Welwyn Garden City, UK). The internal standard, ZN31846, was a gift from Dr Dennis Kyle, Walter Reed Research Center (Bethesda, MD, USA). Acetonitrile was purchased from Burdick and Jackson (Muskegon, MI, USA). All other chemicals were reagent grade and used as purchased.

Preparation of the enantiomers of HF

The enantiomers of HF were resolved by fractional recrystallization of the α-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy)propionic acid diastereomeric complexes according to a previously described method [7].

Achiral chromatography

A Waters 600 E system controller was used with a Waters 484 tunable absorbance detector set at 258 nm (Waters Assoc., Milford, MA, USA), a Shimadzu C-R6A integrator (Shimadzu, Columbia, MD, USA) and a Rheodyne 7125 valve injection system equipped with a 50-μl loop. The 250 × 4 mm i.d. column was packed with 10-μm Lichrosorb RP2 Hibar (Merck, Darmstadt, Germany).

Separation of the internal standard, HF and HFM was performed using a mobile phase of phosphate buffer (10 mM, pH 5)–acetonitrile (25:75, v/v). The analyses were carried out at a flow rate of 1.5 ml min^{-1} and at ambient temperature.

Chiral chromatography

Chiral chromatography was carried out using the system described above for the achiral system. The OVM-CSP phase comprised Ultron ESM-OVM packed in a 150 × 4.6 mm i.d. column) with an Ultron ES-OVM-G guard column (Mac Mod, Analytical Inc., Chadds Ford, PA, USA).

Separation of the enantiomers of HF was accomplished using a mobile phase of phosphate buffer (10 mM, pH 5)–acetonitrile (63:37, v/v). Separation of the enantiomers of HFM required a mobile phase of phosphate buffer (10 mM, pH 5)–acetonitrile (70:30, v/v). The analyses were performed at a flow rate of 1 ml min^{-1} and ambient temperature.

Determination of the elution order of the HF enantiomers. The elution order of the HF enantiomers was determined by chromatography of unequal mixtures of the resolved HF enantiomers. Under the chromatographic conditions used in this study, (−)-HF eluted before (+)-HF.

Sample preparation. The internal standard (15 μl of 0.2 mM ZN31846 in methanol) was added to a 200-μl serum sample followed by 1 ml of *t*-butylmethyl ether. The mixture was acidified by the addition of 40 μl of 0.1 M hydrochloric acid and mixed in a vortex-mixer for 45 s. After centrifugation at 2000*g* for 15 min, the aqueous phase was frozen using dry ice in acetone and the organic phase was decanted. A second extraction of the aqueous phase was carried out with an additional 1 ml of *t*-butyl methyl ether. The organic fractions were pooled and evaporated to dryness; the residue was reconstituted in 70 μl of mobile phase and a 50-μl sample was injected. The mean recovery of HF from spiked serum samples was 95%.

Standard curves. Standard curves for HF and HFM were prepared from standard solutions prepared by the addition of HF and HFM to 800 μl of drug-free plasma. The standard solutions contained the following ng ml^{-1} concentrations of HF–HFM: 25:12.5, 50:25, 100:50, 250:125 and 500:250. Three 200-μl samples were withdrawn from each standard solution and frozen at −20°C until analysis. Standard curves were constructed by plotting the peak area ratios, HF/S and HFM/S, versus HF and HFM concentrations.

Samples for intra-day and inter-day validation studies. Samples containing HF and HFM for the intra-day and inter-day validation studies were prepared in drug-free plasma. The standard solution for the low calibration contained 50 ng ml^{-1} HF and 25 ng ml^{-1} HFM in 3.5 ml of drug-free plasma, and the standard solution for the high calibration contained 500 ng ml^{-1} HF and 250 ng ml^{-1} HFM in 3.5 ml of drug-free plasma. Fifteen 200-μl samples for each data point were withdrawn from the standard solutions and frozen at −20°C until analysis.

Pilot pharmacokinetic study

An oral dose equivalent to 1000 mg of HF base (four 250-mg tablets of Halfan®, SK&F, France) was administered to a healthy male Caucasian volunteer. Blood samples (10 ml) were collected in glass Vacutainers containing EDTA (Beckton Dickinson, NJ, USA) before administration and at 1, 2, 3, 4, 5, 6, 8, 10, 12 h and 1, 2 and 4 days. The samples were separated into two portions just after collection. One 2-ml portion of whole blood was transferred to a polypropylene tube. The remaining sample was centrifuged and the plasma collected and transferred to a separate polypropylene tube. The samples were then stored at −20°C.

Results and Discussion

Achiral chromatography

The molecular structures of HF, HFM and the internal standard (IS) used in this study are presented in Fig. 1. Under the chromatographic conditions used in the achiral system, capacity factors (k') were 5, 8 and 12.3 for IS, HFM and HF, respectively. The chromatograms of an extracted sample of blank plasma, a plasma spiked with the three compounds and a plasma collected 5 h after administration of the drug are presented in Fig. 2(A), (B) and (C), respectively.

In spite of some interference from endogenous plasma components with the chro-

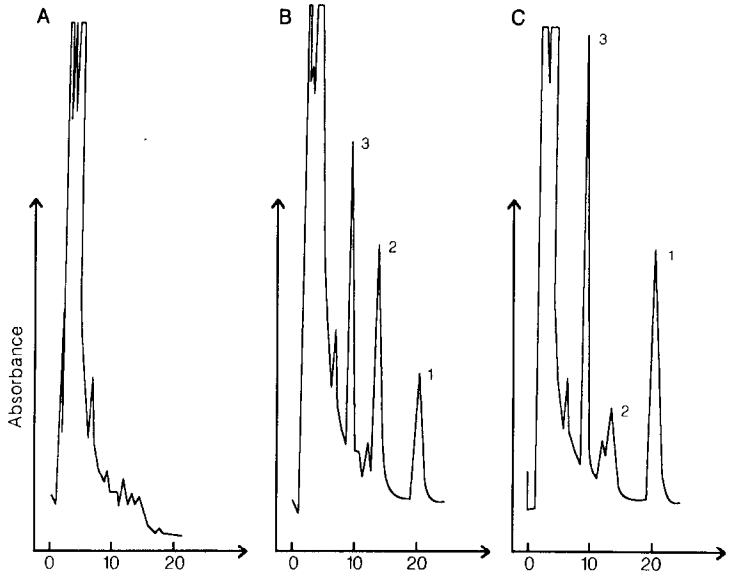

Figure 2
Separation of extracted plasma samples by achiral reversed-phase chromatography. 1, HF; 2, HFM; 3, IS. (A) Blank plasma; (B) plasma spiked with 500 ng ml^{-1} of HF and 250 ng ml^{-1} of HFM; (C) plasma sample 5 h after administration of the drug. Chromatographic conditions: see text.

Table 1
Precision and reproducibility studies for the chromatography of HF and HFM on the achiral HPLC system. See text for chromatographic conditions

Compound	Sample	Spiked concentration (ng ml^{-1})	Mean determined concentration (ng ml^{-1})	RSD (%)
Intra-day (n = 5)				
HF	low	50.0	48.2	9.6
	high	500.0	494.1	9.6
HFM	low	25.0	22.1	7.9
	high	250.0	244.0	7.9
Inter-day (n = 15)				
HF	low	50.0	45.5	8.8
	high	500.0	469.6	9.5
HFM	low	25.0	21.61	6.7
	high	250.0	240.6	7.8

matography of HFM, the standard curves for both HF and HFM were linear over the range investigated. The equations of the curves were $y = 1.77 \times 10^{-3}x - 0.03 \times 10^{-3}$ for HF, and $y = 3.88 \times 10^{-3}x + 0.7 \times 10^{-3}$ for HFM with correlation coefficients of 0.997 and 0.995, respectively.

Since the total drug concentrations were determined on the achiral system, this chromatographic procedure was validated for precision and reproducibility. Table 1 summarizes the intra-day and inter-day reproducibility of the assay for low and high standards of HF and HFM.

Chiral chromatography

Under the chromatographic conditions used for this assay, the k' for (−)-HF was 6.3, the k' for (+)-HF was 15.4, and the k' values for the HFM enantiomers were 5.1 and 10.1. The resolved enantiomers of HFM were not available and the enantiomeric elution order was not determined. The stereoselectivity factors (α) were 1.98 and 2.44 for HFM and HF, respectively. Retention of HF and HFM enantiomers can be decreased by increasing the ionic strength, by decreasing the pH or by increasing the concentration of organic solvent without significantly altering the stereoselectivity [8].

The fractions of HF and HFM were collected from the achiral system, evaporated and injected in the chiral system after dissolution in methanol. The chromatograms of fractions

collected from blank plasma, plasma spiked with the racemic mixture and plasma from the human study (5 h post-dose) are presented in Fig. 3(A), (B) and (C), respectively for HF, and Fig. 4(A), (B) and (C), respectively for HFM. No interfering peaks from the plasma were detected.

Pilot pharmacokinetic study

Plasma and whole blood concentrations were determined over a period of 4 days after the oral administration of 1000 mg of HF (four tablets of Halfan® 250 mg). Total concentrations of HF and HFM were determined by achiral chromatography and the corresponding enantiomeric ratios were calculated from the results of the chromatography on the OVM-CSP. The concentration of each enantiomer was determined using the following equations:

$$\text{total } (+)\text{HF} = [\text{HF}] \times [\%(+)\text{HF}],$$

$$\text{total } (-)\text{HF} = [\text{HF}] \times [\%(-)\text{HF}],$$

$$\text{total } (+)\text{HFM} = [\text{HFM}] \times [\%(+)\text{HFM}],$$

$$\text{total } (-)\text{HFM} = [\text{HFM}] \times [\%(-)\text{HFM}].$$

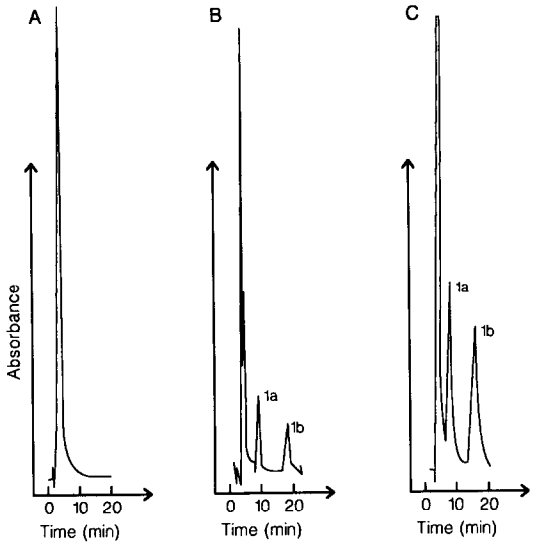

Figure 3
Enantioselective chromatography on the OVM-CSP of eluent fractions collected at the elution time of HF from the chromatography of extracted plasma samples on the achiral system. 1a, (−)-HF; 1b, (+)-HF. (A) Blank plasma; (B) plasma spiked with 250 ng ml^{-1} of HF; (C) plasma sample 5 h after administration of the drug. Chromatographic conditions: see text.

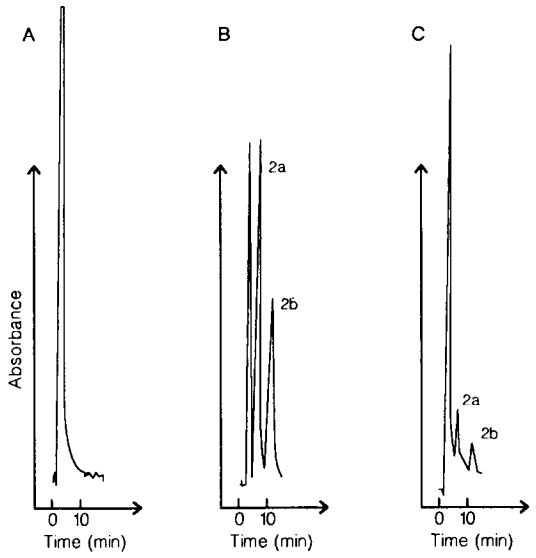

Figure 4
Enantioselective chromatography on the OVM-CSP of eluent fractions collected at the elution time of HFM from the chromatography of extracted plasma samples on the achiral system. 2a, the first eluted HFM enantiomer; 2b, the second eluted HFM enantiomer. (A) Blank plasma; (B) plasma spiked with 250 ng ml^{-1} of HFM; (C) plasma sample 5 h after administration of the drug. Chromatographic conditions: see text.

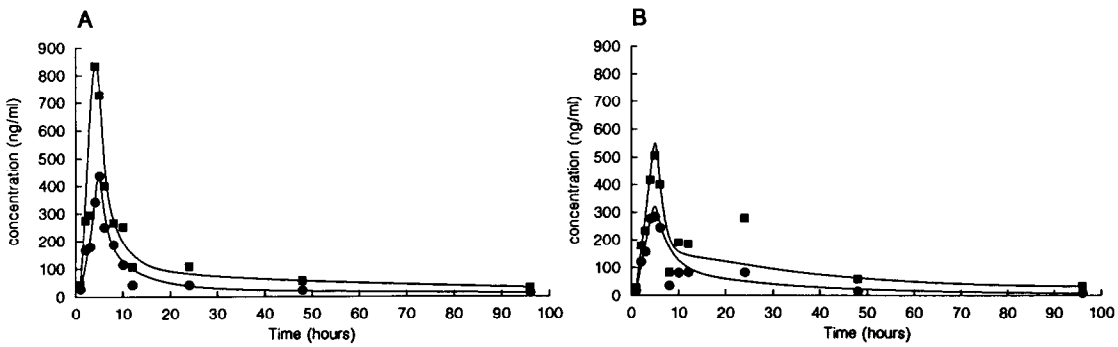

Figure 5
(A) Plasma concentrations of HF enantiomers after administration of 1000 mg of HF base to a healthy volunteer. (B) Whole blood concentrations of HF enantiomers after administration of 1000 mg of HF base to a healthy volunteer. ●, (−)-HF; ■, (+)-HF.

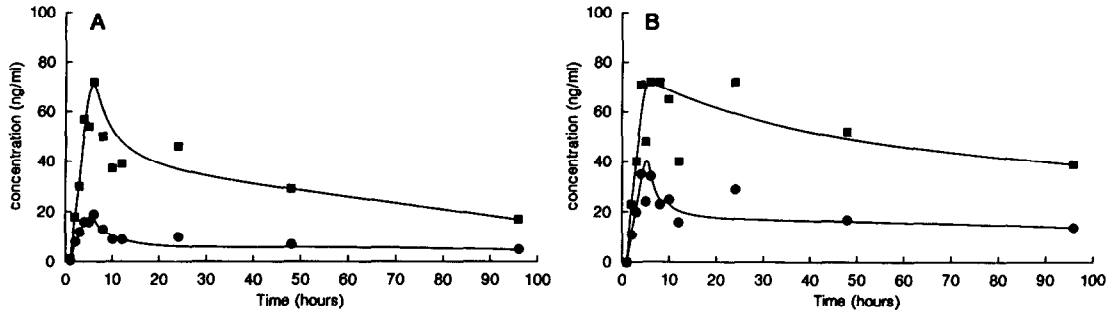

Figure 6
(A) Plasma concentrations of the enantiomers of HFM after administration of 1000 mg of HF base to a healthy volunteer. (B) Whole blood concentrations of HFM enantiomers after administration of 1000 mg of HF base to a healthy volunteer. ●, First eluted enantiomer of HFM; ■, second eluted enantiomer of HFM.

The plasma concentration versus time curves and the whole blood concentration versus time curves for the HF enantiomers are presented in Fig. 5. At all time points, (+)-HF was present in higher concentrations than (−)-HF. In this pilot study, the (+)/(−) ratios in plasma varied from 1.4 to 2.5. The results from the assay of the whole blood samples did not significantly differ from the plasma findings. In whole blood, the (+)/(−) ratios varied from 1.4 to 3.4.

The plasma concentration versus time curves and the whole blood concentration versus time curves for the HFM enantiomers are presented in Fig. 6. The difference between the HFM enantiomers is highly significant. The ratios of the second eluted enantiomer to the first eluted enantiomer in this study varied from 2.2 to 4.5 in plasma and from 2 to 4.2 in whole blood.

From the preliminary results, the pharmacokinetics of HF appears to be stereospecific. This stereospecificity could be due to stereoselective distribution and/or stereoselective metabolism. Investigations to discover the mechanism(s) involved in this process are in progress and the results will be reported elsewhere.

Acknowledgements — This work was supported in part by NCI Grant P30CA21765 and American Lebanese Syrian Associated Charities. The stay of Francois Gimenez at St. Jude Children's Research Hospital was supported in part by the following pharmaceutical companies: Servier, Roussel, Sandoz, Fournier-Dijon, Glaxo, Unicet and Assistance Publique de Paris.

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[*Received for review 24 June 1991*]