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Short communication

Chiral chromatographic method to determine the enantiomers of halofantrine and its main chiral desbutyl metabolite in erythrocytes

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Abstract

We describe a direct liquid chromatographic method with spectrofluorimetric detection to quantify the two enantiomers of halofantrine and the two enantiomers of its main chiral *N*-monodesbutylated metabolite in erythrocyte pellets. The method involves a Chiralpak AD column and a rapid one-step extraction procedure with acetonitrile. The method was validated for the four enantiomers within the range 0–1000 ng/ml. The absence of stereoconversion was studied in samples stored frozen for up to eight months. The optical rotation of the halofantrine and metabolite enantiomers was determined after separation on a semi-preparative Chiralcel OD column with polarimetric detection.

Keywords: Enantiomer separation; Halofantrine; *N*-Desbutylhalofantrine

1. Introduction

Halofantrine (HF) {(+)-1,3-dichloro-α-[2-(dibutylamino)-ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol (Fig. 1)} is a chiral antimalarial agent [1] and is used clinically as the racemic mixture. HF undergoes hepatic metabolism [2,3] with *N*-desbutylhalofantrine (Fig. 1) as the main metabolite. HF is mainly associated with plasma (85%) and erythrocytes (11%) in uninfected subjects, together with lymphocytes, thrombocytes and granulocytes [4]. HF levels in erythrocytes are up to 60-fold higher in patients infected with *Plasmodium falciparum* than in healthy subjects [4]. Given this concentration phenomenon and the fact that antimalarial agents act inside erythrocytes, it is important to be able to quantify HF and its metabolites in red blood cell pellets. Stereoselective studies have demonstrated that the activities of the two HF enantiomers [5,6] and of the two active desbutylhalofantrine hydrochloride (HFM) enantiomers [7,8] are identical.

Several methods have been used to determine halofantrine and its main metabolite in biological fluids but most are not stereoselective and not applied to red blood cells [9–14]. HF has now been separated stereoselectively in solution on a chiral column [15] and in plasma using pre-column derivatization with an enantiopure chiral reagent [16]; HF and its *N*-desbutylated metabolite have been

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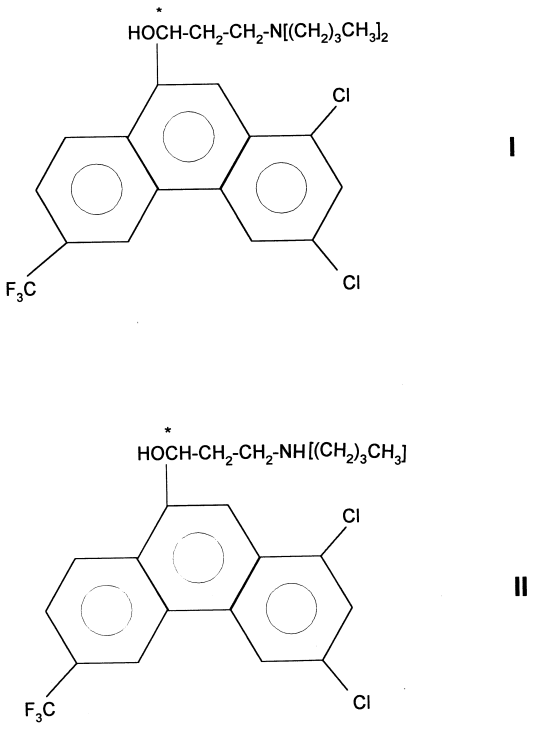

Fig. 1. Chemical structure of halofantrine (I) and its *N*-desbutyl metabolite (II); * asymmetric carbon.

similarly separated in human plasma and whole blood on a chiral ovomucoid column [17] and an amylose Chiralpak AD column [18]. In these last two methods, optical rotation was identified for both HF enantiomers, but the two enantiomers of the *N*-desbutylated metabolite were characterized by the order of elution in the chiral stationary phase.

We describe a method to quantify the enantiomers of both HF and *N*-desbutylated halofantrine in red blood cell pellets after rapid and simple one-step liquid–liquid extraction. We also describe the optical rotation of the two enantiomers of the metabolite.

2. Experimental

2.1. Chemicals

HF, HFM and (±)-dichloro-[2-(dibutylamino) methyl]-6-(trifluoromethyl)-9-phenanthrenemethanol (used as internal standard) were from Smith Kline and Beecham (Welwyn Garden City, UK). HF enantiomers were prepared by fractional crystallization using D-camphoric acid as the chiral reagent [19]. HFM enantiomers were prepared using a semi-preparative Chiralcel OD chromatographic column (250×10 mm, Daicel, Mallinckrodt Baker, Noisy-le-sec, France). Spectroscopy-grade (UV and fluorescent) hexane was from Carlo Erba (Val de Reuil, France). Analytical grade isopropyl alcohol was from Carlo Erba. Analytical grade 2-butanol and diethylamine were from Prolabo (Paris, France). UV-grade acetonitrile was from Merck (Nogent-sur-Marne, France).

2.2. Determination of the optical rotation of HFM enantiomers

The optical rotation of HFM enantiomers was identified using a polarimetric Chiralyser detector (IBZ Mextechnik, ABV France, Saint Germain-en-Laye, France) and a semi-preparative Chiralcel OD column (250×10 mm) (Daicel, Mallinckrodt Baker) with a mobile phase of hexane–2-propanol–diethylamine (90:10:0.1, v/v/v) at a flow-rate of 2 ml/min.

2.3. Analytical chiral chromatography

We used the separation method for HF and HFM enantiomers described by Terefe and Blaschke [18] with slight modifications. The analytical LC system consisted of a Shimadzu LC-6A pump (Touzart et Matignon, Les Ulis, France), a Waters 717 Plus autosampler (Millipore, Saint Quentin, France) and a Shimadzu RF535 spectrofluorimetric detector (Touzart et Matignon) with excitation and emission at 260 nm and 380 nm, respectively. The analytical column was an AD Chiralpak column coated with amylase Tris-3,5-dimethyl phenylcarbamate (250× 4.6 mm I.D., 10 μm) and purchased from (Daicel, Mallinckrodt Baker). The mobile phase was hexane–2-propanol–2-butanol–diethylamine (95:3:2:0.5, v/v) at a flow-rate of 0.3 ml/min at room temperature.

2.4. Extraction from red blood cell pellets

A very simple liquid–liquid extraction method was used. A 1-ml volume of acetonitrile–ethanol

(99:1, v/v) was added to 500 μl of erythrocyte pellet. The mixture was vortexed for 1 min and centrifuged at 2000 *g* for 5 min. The supernatant was collected and evaporated to dryness under nitrogen. The residue was reconstituted in 100 μl of mobile phase and 20 μl was injected into the chiral chromatography column. The extraction procedure was done in silanized tubes (Venoject, Terumo, Saint Quentin, France).

2.5. Samples for the stereoconversion study

Red blood cell pellets were obtained by centrifugation of fresh whole blood at 3000 *g* for 15 min and by collecting the red blood cells from the bottom of the tube, far from the interface to avoid contamination by other cells.

To confirm the absence of stereoconversion of HF and HFM enantiomers in biological samples, red blood cell samples were spiked with various ratios of isomers (in the racemic condition: 50:50 and in a different (±) proportion: 71:29) of HF and HFM. Each sample was tested in duplicate on days 0, 1, 56 and 240.

2.6. Validation

The method was validated for the four separated enantiomers, i.e., (+)-HF; (−)-HF; (+)-HFM and (−)-HFM. The second eluted enantiomer of the chiral internal standard was used for quantification. Standard curves were prepared by adding various amounts of rac-HF and rac-HFM to red blood cell pellets to produce the following concentrations of each enantiomer: 50, 100, 250, 500, 750 and 1000 ng/ml. Within-assay precision was determined by analyzing five samples spiked with (+)-HF, (−)-HF, (+)-HFM and (−)-HFM at concentrations of 100 ng/ml and 750 ng/ml. Between-assay precision was determined by analyzing spiked samples on three consecutive days.

The extraction recovery of HF and HFM was estimated from the ratio between the peak area obtained with an extracted spiked sample and the peak area obtained after direct injection of the same amount.

3. Results and discussion

3.1. Determination of optical rotation and order of elution of the HF and HFM enantiomers

The enantiomer elution order was determined by chromatographing the separated enantiomers on the Chiralcel OD and the Chiralpak AD stationary phases. It is interesting to note that the elution order of the HF enantiomers was identical on the Chiralcel OD and Chiralpak AD stationary phases and that the elution order of HFM enantiomers was reversed on Chiralcel OD and Chiralpak AD.

3.2. Chromatography

Fig. 2 shows typical chromatograms obtained after extraction of a sample spiked with racemic HF and racemic HFM and a sample from a malaria patient 14 h after oral administration of 750 mg of racemic halofantrine. For the patient, (+)-HF concentration is slightly higher than (−)-HF concentration. No difference is observed for the concentrations of HFM enantiomers.

3.3. Standard curves, recovery, reproducibility and accuracy

Over the three days of the study, standard curves were linear in the range of 0 to 1000 ng/ml for the four compounds studied, with a minimum correlation coefficient of 0.994.

Recovery, accuracy and within-day and between-day precision are presented in Table 1. For the four compounds, within-day precision was less than 14.9% and between-day precision was less than 19.8% for the low and high concentrations.

Over the validation period, mean HF and HFM recovery was 57.0±5.1% and 40.0±3.7%, respectively. The limit of quantification, defined as the minimum concentration with a standard deviation below 25%, was 25 ng.

3.4. Stereoconversion

The ratios obtained with samples spiked with different amounts of each of the enantiomers on days 0, 1, 56 and 240 show the absence of stereoconver-

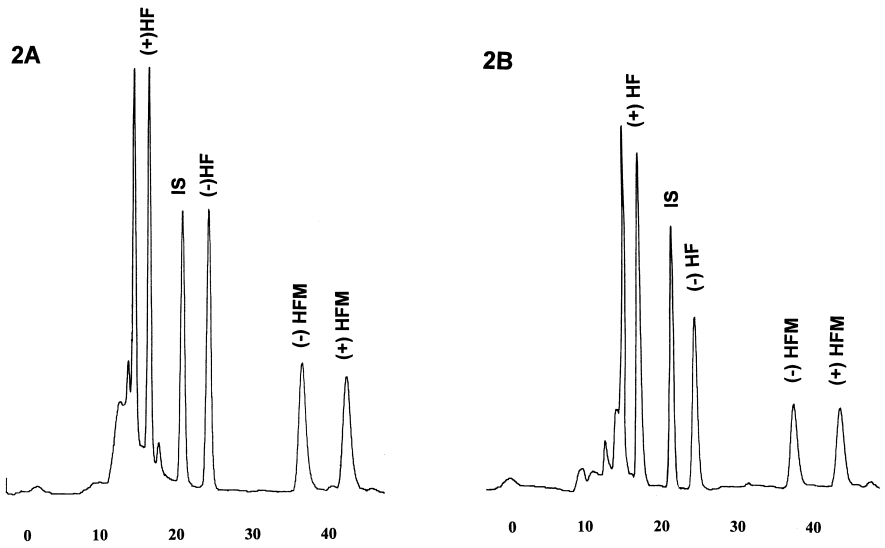

Fig. 2. Representative chromatograms of an extracted sample spiked with (+)-HF (250 ng/ml), (−)-HF (250 ng/ml), (+)-HFM (250 ng/ml) and (−)-HFM (250 ng/ml) (2A) and an extracted sample from a patient 14 h after oral administration of 750 mg of racemic halofantrine (2B).

Table 1
Within-day precision and between-day (three days) precision for halofantrine enantiomers and *N*-desbutylhalofantrine enantiomers in spiked red blood cell pellets

Compound	Concentration (ng/ml)	Precision (%)	
		Between assay	Within-assay
(+)-HF	100	14.7	4.7
	750	8.4	3.1
(−)-HF	100	5.8	3.0
	750	7.4	2.9
(+)-HFM	100	16.0	14.9
	750	14.6	4.3
(−)-HFM	100	19.8	9.9
	750	14.7	6.0

sion and that biological samples may be stored at −20°C for at least eight months before chiral chromatographic analysis.

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