ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 911–918

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

A liquid chromatographic assay for the stereospecific quantitative analysis of halofantrine in human plasma

Dion R. Brocks *, Michael J. Dennis, William H. Schaefer

Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals Research and Development, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406, USA

Received for review 5 July 1994; revised manuscript received 14 October 1994

Abstract

A stereospecific liquid chromatographic (LC) assay was developed for the quantification of the antimalarial drug, halofantrine, in human plasma. Following protein precipitation with acetonitrile, the enantiomers of halofantrine were extracted from human plasma using ammonium hydroxide and *tert*-butyl methyl ether–hexane. A precolumn derivatization step was employed using (+)-di-*O*-acetyl-L-tartaric acid anhydride to form diastereomeric derivatives of the halofantrine enantiomers. Chromatographic resolution of the diastereomers was performed using reversed-phase LC with UV detection at 254 nm. The recovery of (±)-halofantrine from human plasma at 25 and 2000 ng ml^{-1} was 68.2 and 61.4%, respectively. The derivatization yield following extraction and derivatization of 2000 ng ml^{-1} of (±)-halofantrine was 95.6%. Using 0.5 ml of plasma, the limit of quantification for each halofantrine enantiomer was 12.5 ng ml^{-1}. Linear responses in analyte/internal standard peak height ratios were observed for analyte concentrations ranging from 12.5 to 1000 ng ml^{-1}. Chromatograms of drug-free plasma showed no interfering peaks with retention times similar to those for (+)- and (−)-halofantrine or internal standard. Based on the validation data, the assay performed well over the enantiomer concentration range of 12.5–500 ng ml^{-1}.

Keywords: Halofantrine enantiomers; Malaria; Reversed-phase chromatography

1. Introduction

Halofantrine (SK&F 102886; (±)-1,3-dichloro-α-[2-(dibutylamino)-ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol; Fig. 1) is an effective agent against multi-drug resistant *Plasmodium falciparum* infections [1]. Similar to other antimalarial drugs, such as chloroquine, hydroxychloroquine, mefloquine, primaquine, and quinacrine, halofantrine possesses an alkyl side chain containing amino and hydroxyl functional groups. Each one of these agents possesses a chiral centre on the alkyl side chain, and all are administered clinically as the racemate.

Stereoselectivity in the pharmacokinetics of antimalarial drugs has previously been documented [2–5]. Most of the assays used in these studies have involved the use of LC with chiral stationary phases for the resolution of the enantiomers [3,5,6]. There are two such assays currently published which describe the quantitative analysis of halofantrine enantiomers in biological specimens [7,8]. One of the assays, which is intended for use in pharmacokinetic studies using plasma or blood, requires prior fractionation of (±)-halofantrine using non-stereospecific chromatography, followed by stereospecific chromatography using an ovomucoid

* Corresponding author. Tel: (610)-270-5594.

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SSDI 0731-7085(95)01343-1

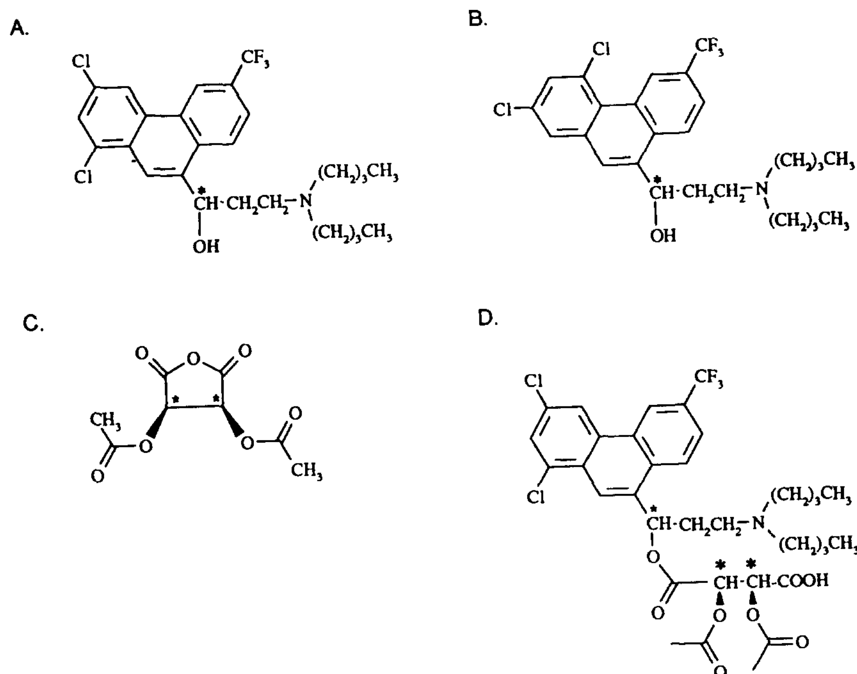

Fig. 1. Structures of halofantrine (A), internal standard (B), DATAAN (C), and proposed structure for derivatized halofantrine (D). Assymmetric centers are denoted by *.

chiral stationary phase column [7]. Another procedure has also recently been published, which uses a chiral stationary phase without the need for non-stereospecific chromatography and fraction collection [8].

In a recently published assay the use of precolumn derivatization with an enantiopure chiral reagent, (±)-di-*O*-acetyl-L-tartaric acid anhydride (DATAAN), was reported in an assay for hydroxychloroquine enantiomers in human specimens [9]. The assay achieved chromatographic baseline resolution of the derivatized hydroxychloroquine enantiomers, using a procedure that was less time consuming than another method requiring the use of achiral/chiral sequential chromatography [6]. This report describes a sensitive stereospecific LC method for the assay of halofantrine enantiomers in human plasma using precolumn derivatization with DATAAN, and chromatographic resolution of the formed halofantrine diastereomers using a conventional C_{18} column.

2. Experimental

2.1. Chemicals

Racemic halofantrine HCl (Fig. 1), monodesbutylhalofantrine and internal standard (SK&F 99123; Fig. 1) were supplied by SmithKline Beecham Pharmaceuticals. The individual enantiomers of halofantrine were kindly provided by the Walter Reed Army Hospital, Department of Experimental Therapeutics. Analytical grade dichloromethane, potassium phosphate (monobasic), triethylamine, and LC grade acetonitrile, hexane, water, methanol and *tert*-butyl methyl ether, were purchased from Baker Laboratories (Phillipsburg, NJ, USA). Sulfuric and glacial acetic acids, and ammonium hydroxide were purchased from Malinckrodt (Paris, KY, USA). DATAAN (Fig. 1), of claimed purity > 97%, was purchased from Fluka (Ronkonkoma, NY, USA). Water for the LC mobile phase was obtained using a Hewlett-Packard 661A water purifier.

2.2. Chromatography

A Hewlett-Packard 1050 integrated LC system (Hewlett-Packard, Palo Alto, CA, USA) comprising a pump, injector, and UV detector was used for chromatographic analysis. The UV wavelength was set at 254 nm. The chromatographic separations of halofantrine, the internal standard (IS), and their diastereomers, were accomplished using a 4.6 mm i.d. × 25 cm Ultrasphere-ODS column (Beckman, Fullerton,

CA, USA) attached to a Guard-Pak Precolumn Module (Waters Millipore, Milford, MA, USA) containing an ODS cartridge insert. The chromatographic output was stored and integrated using the Access* Chrom Data Acquisition computer software package (PE Nelson, Cupertino, CA, USA).

For the mobile phases, a solution of 0.025 M potassium phosphate (monobasic) containing 1.5 ml of 2 M sulfuric acid and 0.5 ml of triethylamine per liter (pH $\approx$ 5.0) was prepared. The mobile phase used for the stereospecific analysis was a 46.5:53.5 (v/v) mixture of 0.025 M potassium phosphate solution–acetonitrile, containing 0.9 g l^{-1} sodium dodecyl sulfate, pumped at a flow rate of 1.2 ml min^{-1}. For the non-stereospecific chromatography used in the recovery and derivatization yield experiments, the mobile phase consisted of a 25:75 (v/v) mixture of 0.025 M potassium phosphate solution-acetonitrile, containing 1.5 g l^{-1} sodium dodecyl sulfate, pumped at 1.5 ml min^{-1}. All mobile phases were degassed by filtering them through a 0.45 μm filter. Chromatographic separations were carried out at room temperature.

2.3. Stock solutions

A 100 μg ml^{-1} stock solution of halofantrine was prepared by dissolving 10.7 mg of racemic halofantrine HCl in 100 ml of 20% LC water in acetonitrile. To prepare the samples for the calibration curves and assessment of validation, working solutions of 10, 1, and 0.1 μg ml^{-1} of racemic halofantrine were prepared by making successive 1/10 dilutions of the working stock solution with acetonitrile. The stock solution of IS was prepared by weighing and dissolving 10 mg of IS in 100 ml of 20% LC water in acetonitrile. The working IS solution was made by diluting the stock solution by a factor of 10.

For derivatizing the halofantrine enantiomers, a 0.25 M solution of DATAAN was freshly prepared in acetic acid–dichloromethane (1:4, v/v). All stock solutions were refrigerated at 4 °C in an amber bottle between use.

2.4. Sample preparation

To 0.5 ml of human plasma was added 30 μl of a 100 μg ml^{-1}stock solution of IS. While being vortex mixed at high speed, 2 ml of acetonitrile was carefully added to each tube to precipitate the plasma proteins. The tubes were subsequently centrifuged at approximately 1800*g* for 3 min. After carefully transferring the supernatant to new test tubes, 0.5 ml of ammonium hydroxide and 5 ml of *tert*-butyl methyl ether–hexane (1:1, v/v) were sequentially added. The tubes were vortex mixed for 90 s at high speed, and then centrifuged for 5 min at 1800*g*. The uppermost organic layer was carefully transferred to new tubes and evaporated to complete dryness under nitrogen at 25 °C.

To the extraction residue was added 300 μl of DATAAN solution. The tubes were covered and placed in an oven at 45 °C for 30 min. Methanol (0.3 ml) was added to each tube to react with excess reagent, and the solvent was evaporated to dryness under nitrogen at 25 °C. The dried derivatized extracts were reconstituted in 170 μl of mobile phase and transferred to glass inserts. Volumes of 30–100 μl of each sample were injected into the LC apparatus.

2.5. Extraction efficiency

The extraction efficiency was calculated by adding known amounts of (±)-halofantrine to 0.5 ml of drug-free plasma and extraction into *tert*-butyl methyl ether–hexane (1:1, v/v) as described above. After evaporation of the organic solvent the samples were reconstituted with 0.2 μl of the mobile phase and equal volumes injected into the LC system using the method for non-stereospecific chromatography. The peak areas were compared to those obtained from equivalent volumes of standard solutions of halofantrine which were evaporated to dryness, reconstituted with 0.2 ml of mobile phase, and directly injected into the LC system. The concentrations used to assess extraction efficiency were 25 and 2000 ng ml^{-1} of the racemate, each determination being performed in quadruplicate.

2.6. Derivatization yield

Plasma samples ($n = 8$) spiked with (±)-halofantrine (2000 ng ml^{-1}) and IS (600 ng ml^{-1}) were subjected to the protein precipitation and extraction steps outlined above. Four of the dried extracts were then derivatized as described above, while the remaining four were set aside. After the derivatization step was completed, the dried residues from each of the tubes were reconstituted with 200 μl of the mobile

phase and equal volumes injected into the LC system employing the method for non-stereospecific analysis. The derivatization yields of halofantrine and IS were estimated from the difference in the amount of the underivatized species present between the samples.

2.7. Calibration, accuracy and precision

Six replicate plasma samples containing 12.5, 25, 100, and 500 ng ml^{-1} of each halofantrine enantiomer were extracted and analyzed on three separate days. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. The calibration curves were comprised of plasma samples containing 12.5, 25, 50, 100, 250, 500, and 1000 ng ml^{-1} of each halofantrine enantiomer. Based upon analysis of residuals, a weighted fit ($1/y$) linear regression was used to construct a calibration curve for the peak height ratio of analyte to the first diastereomeric peak of IS vs. analyte concentration. Intraday accuracy was estimated based on the mean percentage error (100 × (mean measured concentration/expected concentration)), and the interday accuracy was calculated as the mean of the intraday accuracy determinations. The precision, expressed as a percentage, was evaluated by calculating the intra- and inter-day relative standard deviations (RSDs).

2.8. Assessment of order of elution

A small amount of each enantiomer was added to two separate test tubes. 0.5 ml of LC grade water, 50 μl of 2 M NaOH and 5 ml of *tert*-butyl methyl ether–hexane were added, after which the tubes were vortex mixed for 30 s and centrifuged for 3 min at 1800*g*. The organic layer was transferred to new tubes, and the solvent evaporated to dryness under nitrogen. The samples were derivatized with DATAAN as described above, and the final dried extract reconstituted with 200 μl of the mobile phase. The enantiomeric purity of the (+) and (−) enantiomers and the order of elution of the derivatized halofantrine enantiomers were established, comparing retention times and peak areas.

2.9. Assessment of derivative structure by mass spectrometry

(±)-Halofantrine stock solution (0.5 ml of 100 μg ml^{-1}) was added into each of two test tubes containing 50 μl of 2 M NaOH. After adding 5 ml of *tert*-butyl methyl ether–hexane (1:1, v/v), the samples were vortex mixed for 30 s, centrifuged for 5 min, and the organic layer transferred to clean tubes and evaporated to dryness. Derivatization of one of the tubes was performed as described above using 0.5 ml of 0.25 M DATAAN solution, followed by the addition of 0.5 ml of methanol and evaporating to dryness. The mass spectral analyses were performed with a Sciex API III mass spectrometer (Perkin-Elmer/Sciex, Toronto, Canada) with Ionspray ionization (a proprietary, pneumatic nebulizer-assisted electrospray ionization interface) operated in the positive-ion mode. Halofantrine and its derivative at a concentration of approximately 10 μg ml^{-1}, were infused into the instrument at a flow rate of 5 μl min^{-1} using a mobile phase consisting of 2:1 (v/v) acetonitrile–ammonium acetate (pH 5.0; 10 mM). The ionspray potential was 4500 V and the interface potential was 650 V for all experiments. The oriface potential was set to 80 V and product-ion experiments were performed using argon as a collision gas at a thickness setting of 200×10^{12} atoms cm^{-2} and a collision energy of 27.5 V. Mass spectra used to obtain molecular weight information were acquired over a range of 150–1000 mass units at a rate of 2–4 s per scan. Product ion mass spectra were obtained at a similar scan rate from 50 mass units over a range which included the parent ion. Data from five to eight scans were averaged for each mass spectrum.

2.10. Pharmacokinetic evaluation

The plasma samples from a healthy male volunteer (20 years old, 86.4 kg), who had been given 500 mg of a halofantrine HCl suspension, were analyzed using the stereospecific assay procedure. The blood samples were collected in heparinized polypropylene tubes and the plasma stored at −25 °C until being analyzed. The area under the plasma concentration vs. time curve (AUC) from time 0 to 32 h was determined using the combined log–linear trapezoidal rule, and C_{max} and T_{max} were determined by visual inspection of the data.

3. Results and discussion

In the chromatograms from the stereospecific analysis (Fig. 2) the derivatized halofantrine

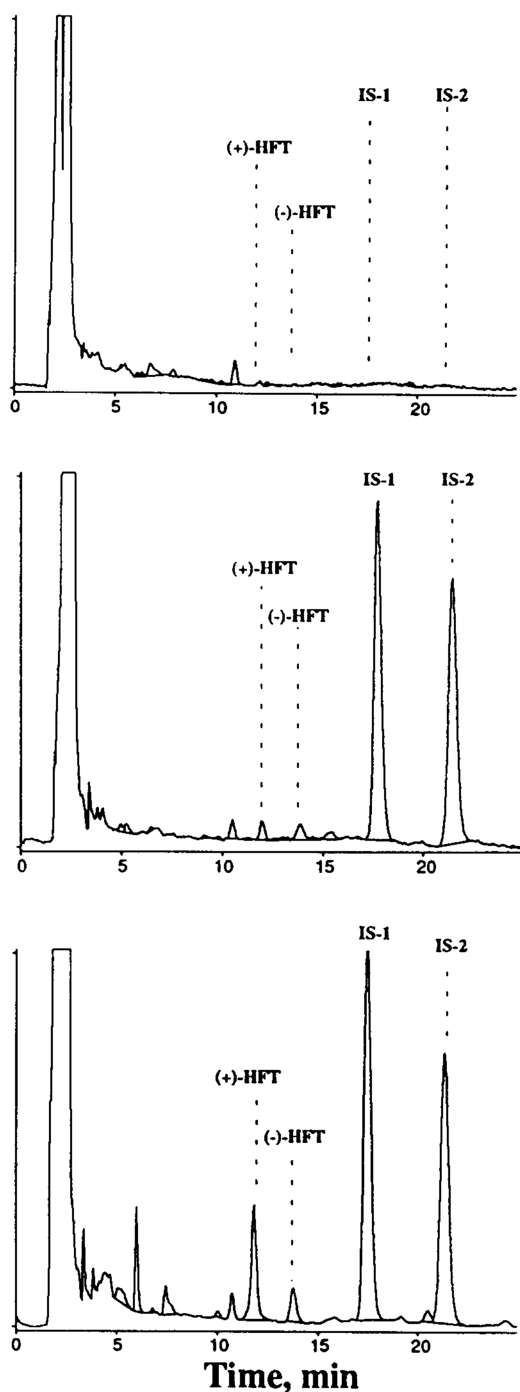

Fig. 2. Chromatograms of drug-free human plasma (top), plasma spiked with 12.5 ng ml^{-1} of (+)- and (−)-halofantrine (middle), and plasma from a healthy male volunteer obtained 32 h after taking an oral dose of 250 mg halofantrine (bottom). Abbreviations: HFT, halofantrine: IS, internal standard.

enantiomers eluted chromatographically at 11.5 and 13.3 min, whereas the diastereomers of the IS eluted at 17.0 and 20.7 min. Baseline resolution of each diastereomer pair was achieved (Fig. 2), and the peaks were symmetrical in appearance and free from interference from endogenous components in plasma. Using the authentic enantiomer samples, the diastereomer peak corresponding to the (+) enantiomer was found to elute before that of the (−) enantiomer. There was less than 0.6% enantiomeric impurity in the sample of (−)-halofantrine, and less than 0.11% enantiomeric impurity in the (+) enantiomer sample. Hence, the maximum degree of racemization possible under the conditions employed during sample preparation was less than 0.6%.

The column capacity factors (k') were 6.0 and 7.4 for derivatized (+)- and (−)-halofantrine, respectively, and 9.4 and 11.8 for the first and second internal standard diastereomers, respectively. The column selectivity factors (α) for the halofantrine and IS diastereomers were 1.23 and 1.26, respectively. Resolution factors of 2.3 and 3.6 were calculated for the diastereomers of halofantrine and internal standard, respectively.

The non-stereospecific chromatographic conditions used for the determination of extraction and derivatization yield resulted in halofantrine and IS peaks free from interference from endogenous components in plasma. The elution times of (±)-halofantrine and (±)-IS were 11.6 and 16.8 min, respectively. The estimated mean recovery of (±)-halofantrine from human plasma was 68.2 and 61.4% at 25 and 2000 ng ml^{-1}, respectively. After extraction of 2000 ng ml^{-1} of (±)-halofantrine from human plasma, the mean derivatization yield was estimated at 95.6%. The corresponding yield for the IS was 96.9%.

Linear responses were observed in the analyte/IS peak height ratio for enantiomer concentrations ranging from 12.5 to 1000 ng ml^{-1}. Correlation coefficients obtained using weighted ($1/y$) linear regression analysis of calibration curves were typically 0.999 for each enantiomer. Typical equations describing the regression lines were $y = 0.00402x + 0.0050$ and $y = 0.0031x - (2.0 \times 10^{-6})$ for (+)- and (−)-halofantrine, respectively, where y represents the analyte/IS peak height ratio and x represents the plasma concentration. The validation data showed the assay to be sensitive, accurate and precise, with a mean interday error and an RSD of less than 15% and 10%, respectively (Table 1). Using 0.5 ml of human plasma the validated limit of quantification for each halofantrine enantiomer was 12.5 ng ml^{-1}.

Table 1
Accuracy and precision data for (+)- and (−)-halofantrine enantiomers in human plasma

Nominal concentration (ng ml^{-1})		Run 1		Run 2		Run 3		Average within-run precision		Between-run precision		Average accuracy	
		(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
12.5	Mean	10.5	10.5	10.5	12.1	11.5	10.7	8.59	9.96	5.41	7.85	86.7	89.1
	SD	0.68	0.90	0.67	1.39	1.49	1.06						
	N	6	6	6	6	6	6						
	RSD(%)	6.51	8.55	6.36	11.5	12.9	9.86						
	Accuracy	84.0	84.4	84.0	97.1	92.1	85.8						
25	Mean	24.2	23.6	23.5	24.7	24.2	24.5	5.34	8.44	1.74	2.54	95.9	97.0
	SD	1.34	2.05	1.24	2.19	1.26	1.90						
	N	6	6	6	6	6	6						
	RSD(%)	5.54	8.68	5.26	8.86	5.21	7.76						
	Accuracy	96.9	94.2	93.9	98.8	96.7	98.1						
100	Mean	97.8	95.2	102.6	100.2	98.0	101.1	2.04	6.31	2.73	3.18	99.5	98.8
	SD	1.67	3.60	1.50	10.4	2.89	4.82						
	N	6	6	6	6	6	6						
	RSD(%)	1.71	3.78	1.46	10.4	2.95	4.77						
	Accuracy	97.8	95.2	102.6	100.2	98.0	101.1						
500	Mean	503.1	503.2	505.9	505.7	517.3	516.5	2.38	4.05	1.47	1.39	101.8	101.7
	SD	8.88	13.9	9.53	14.8	18.1	33.4						
	N	6	6	6	6	6	6						
	RSD(%)	1.76	2.76	1.88	2.93	3.50	6.46						
	Accuracy	100.6	100.6	101.2	101.1	103.5	103.3						

Mass spectrometry was used to confirm the proposed halofantrine derivative structure (Fig. 3). Underivatized halofantrine displayed an intense $[M+H]^+$ signal at m/z 500 with an isotope pattern consistent with the presence of two chlorine atoms. Collisionally induced dissociation of halofantrine (m/z 500) yielded an abundant ion at m/z 482 resulting from the elimination of water, and product ions at m/z 142, 100, and 58 corresponding to cleavages of the amine side chain with charge retention on the amine. Dehydrated halofantrine (m/z 482), formed prior to Q1 using an orifice potential of 120 V, also yielded product ions at m/z 142, 100, and 58, indicating that these ions could be formed following the elimination of water from halofantrine. Following derivatization, an $[M+H]^+$ appeared at m/z 716 with an isotope pattern consistent with the presence of two chlorine atoms (Fig. 3, inset). This $[M+H]^+$ was consistent with the addition of one molecule of di-O-acetyl-L-tartaric acid to halofantrine. Collisionally induced dissociation of the m/z 716 ion yielded a product ion at m/z 482. This ion was due to the elimination of di-O-acetyl-L-tartaric acid from the derivative,

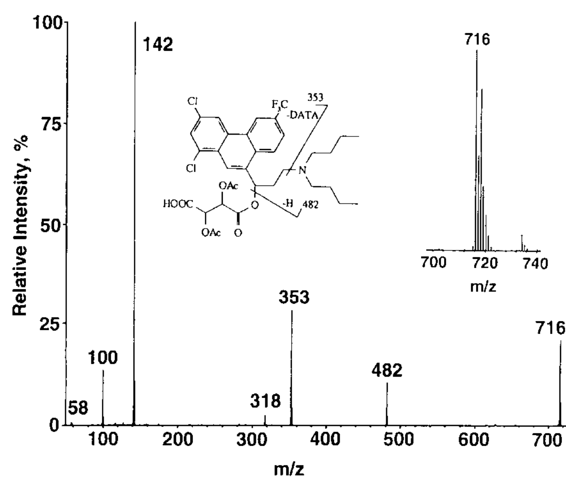

Fig. 3. Mass spectra showing the $[M+H]^+$ for the halofantrine/DATAAN derivative (insert) and the product ions formed from collisionally induced dissociation of the m/z 716 ion. DATA corresponds to di-O-acetyl tartaric acid.

analogous to that resulting from the elimination of water from halofantrine. An additional product ion was observed at m/z 353, which resulted from elimination of both di-O-acetyl-L-tartaric acid and dibutylamine. Product ions at m/z 142, 100, and 58 similar to halofantrine

were also formed. Collisionally induced dissociation of the ion at m/z 718 corresponding to the mono-^{37}Cl isotope yielded product ions at m/z 484 and 355 (but not at m/z 482 and 353) confirming the presence of two chlorine atoms in these product ions, as well as ions at 142, 100, and 58 which did not contain chlorine.

The assay of monodesbutylhalofantrine, the major metabolite of halofantrine, was not possible by the described procedure owing to the formation of four chromatographic peaks after derivatization with DATAAN. These peaks eluted within the first 7 min using the stereospecific mobile phase, and did not interfere with halofantrine or the IS. Although monodesbutylhalofantrine possesses antimalarial activity [1], it is apparently not directly responsible for the prolongation of the QTc interval associated with the drug [10].

Two other LC stereospecific procedures are available for the quantitative analysis of halofantrine in human plasma [7,8], both of which possess the advantage of resolving the enantiomers of monodesbutylhalofantrine. However, one of the methods involves a considerable expenditure of time, owing to its need for quantitative non-stereospecific chromatography of total (±)-halofantrine and (±)-monodesbutylhalofantrine, followed by fraction collection and injection of the dried/reconstituted eluent fractions into a ovomucoid chiral column [7]; the present method took 2.5 h to prepare 31 plasma samples. Further, because halofantrine is generally used in economically impoverished areas of the world, cost is a relevant concern in generating an assay for its plasma concentration monitoring. The costs of both alternative stereospecific methods [7,8] are significantly increased owing to their use of relatively expensive chiral columns. DATAAN, in contrast, is inexpensive, and a 50 g bottle is sufficient for the analysis of over 3000 plasma samples.

As reported previously [7], stereoselective pharmacokinetics were observed in the plasma samples from the healthy subject given a single oral dose of halofantrine (Fig. 4). The values of C_{max} were 119.6 and 68.2 mg ml^{-1} for (+)- and (−)-halofantrine, respectively, whereas T_{max} occurred at 4 h for both enantiomers. The AUC from time 0 to 32 h postdose was 2161 and 752 ng h ml^{-1} for the (+) and (−) enantiomers, respectively.

Although the enantiomers of halofantrine possess similar inhibitory properties towards *P. falciparum* in vitro [11], stereoselective pharmacokinetics could influence the relative in vivo pharmacological activities of the enantiomers. The preliminary evidence available to date indicates that stereospecific analysis of plasma samples may be helpful in accurately defining the pharmacokinetics and pharmacodynamics of halofantrine.

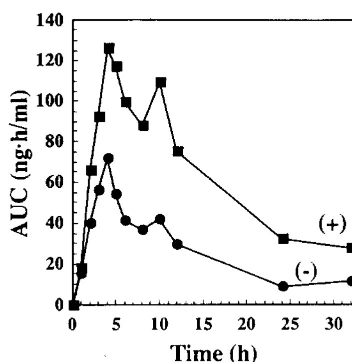

Fig. 4. Halofantrine enantiomer plasma vs. time profiles from a healthy subject given a single oral dose of 500 mg racemic halofantrine HCl: squares, (+)-halofantrine; circles, (−)-halofantrine.

Acknowledgments

The authors extend their thanks to Drs. Dave Wesche and Bill Ellis of the Division of Experimental Research, Walter Reed Army Institute, for supplying the authentic enantiomers used in this study, and Drs. John Horton, Colin Broom, and Sandra Eagle of SmithKline Beecham for their participation in the clinical aspect of the study.

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