*Journal of Chromatography*, 433 (1988) 339–344
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4406

## Note

# Determination of halofantrine and its principal metabolite desbutylhalofantrine in biological fluids by reversed-phase high-performance liquid chromatography

K. ASHLEY MILTON

*Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX (U.K.)*

STEPHEN A. WARD

*Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool L3 5QA (U.K.)*

and

GEOFFREY EDWARDS*

*Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX (U.K.)* and Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool L3 5QA (U.K.)*

(First received June 15th, 1988; revised manuscript received July 25th, 1988)

The emergence of resistant strains of *Plasmodium falciparum* [1] has prompted an evaluation of alternative drugs for malaria prophylaxis and treatment. Halofantrine, 1,3-dichloro-[2-(dibutylamino)ethyl]-6-(trifluoromethyl)-9-phenanthrenemethanol (Fig. 1), was synthesised originally by Colwell et al. [2] as one of a series of arylaminopropanols. It appears to be effective in vivo against multidrug resistant *P. falciparum* [3,4]. In order that the drug may be used optimally in malaria treatment, a detailed knowledge of the clinical pharmacology of halofantrine is required. This demands that suitable sensitive and selective analytical methods are available. Hines et al. [5] reported a high-performance liquid chromatographic (HPLC) method for the analysis of halofantrine in whole blood but this is not ideal due to a relatively high limit of sensitivity (50 $\mu g\ l^{-1}$) and poor selectivity.

This paper describes an improved HPLC method for the determination of both halofantrine and the pharmacologically active metabolite, desbutylhalofantrine, in plasma, packed red cells and urine and its initial application to a study of the disposition of the drug in man.

0378-4347/88/$03.50 

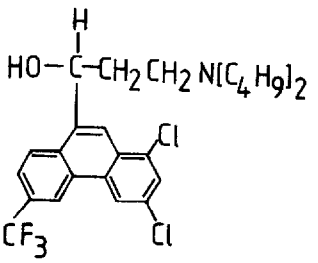


Fig. 1. Structure of halofantrine.

## EXPERIMENTAL

### *Chemicals*

Halofantrine hydrochloride, desbutylhalofantrine hydrochloride and the internal standard, 1,3 dichloro-[2-(dibutylamino)methyl]-6-(trifluoromethyl)-9-phenanthrenemethanol hydrochloride, were gifts from SK & F Research (Welwyn, U.K.). The compounds were synthesised by the Walter Reed Army Institute of Research (Washington, DC, U.S.A.). Analytical-grade dimethyldichlorosilane, orthophosphoric acid and ammonia (0.88 sp.gr) were obtained from BDH (Poole, U.K.). All other reagents were of HPLC grade (Fisons, Loughborough, U.K.). Glass culture tubes and PTFE-lined screw-caps were supplied by Oakes Eddon (Liverpool, U.K.). $C_8$ Bond-Elut solid-phase extraction cartridges (500 mg sorbent mass, 2.8 ml column volume) were obtained from Jones Chromatography (Hengoed, Mid-Glamorgan, U.K.).

### *Chromatography*

The method was developed on a Spectra-Physics liquid chromatograph. The system consisted of an SP8800 ternary HPLC pump equipped with a Rheodyne valve injection system and was coupled to an SP8450 UV–VIS variable-wavelength absorbance detector operating at 254 nm. Chromatographic separation was achieved at ambient temperature on a $C_8$ (Spherisorb; 5 $\mu$m particle size) 10 cm$\times$0.6 cm reversed-phase cartridge Universal Ferruleless column (Capital HPLC Specialists, Bathgate W. Lothian, U.K.). The mobile phase, mixed online, consisted of acetonitrile–water–methanol (78:10:12, v/v) containing triethylamine (1%) buffered to pH 6.0 with orthophosphoric acid and flowing at 1.0 ml min$^{-1}$.

### *Extraction procedure*

*Plasma and packed red blood cells.* The extraction was carried out in 10-ml capacity glass culture tubes pretreated with dichlorodimethylsilane in toluene (5%, v/v) in order to minimise non-specific adsorption to active sites on the glass. To samples of plasma or red blood cells were added acetonitrile (1.0 ml) to precipitate proteins and internal standard (100 or 200 ng; 10 or 20 $\mu$l of a stock solution in methanol as described below). The mixture was vortex-mixed (30 s) and centrifuged (3000 *g* for 10 min). The supernatant was transferred to a Bond-

Elut $C_8$ solid-phase extraction cartridge preconditioned with acetonitrile (1 ml). The supernatant was drawn through the cartridge under vacuum (Vac-Elut SPS 24, Jones Chromatography). The cartridges were then washed with acetonitrile ($2 \times 1.0$ ml). Halofantrine, the desbutyl metabolite and internal standard were eluted from the cartridge with a mixture of 1.0 *M* hydrochloric acid–acetonitrile (1:9, v/v, 1 ml) and the eluent was collected into clean silanised tubes. Ammonia (1.0 ml, 0.88 sp. gr) and dichloromethane (5.0 ml) were added to the eluent. Mechanical tumbling (Stuart tube-rotator, TR-2) of the mixture (10 min) was followed by centrifugation (2000 *g* for 10 min) and the organic phase was carefully removed and evaporated to dryness in a water-bath under a steady stream of nitrogen gas at 37 °C. The sample was then reconstituted in methanol (20 μl) and an aliquot (10–20 μl) injected on to the column.

*Urine.* Urine (100 μl) was placed in 10-ml capacity glass tubes together with the internal standard (200 ng; 20 μl as a solution in methanol), ammonia (900 μl, 0.88 sp. gr) and dichloromethane (5.0 ml). Sample preparation was as described as above except that solid-phase extraction was omitted.

*Calibration*

Stock solutions of halofantrine, desbutylhalofantrine (1 and 10 mg $l^{-1}$) and the internal standard (10 mg $l^{-1}$) were prepared by dissolution of each substance in methanol and stored at 4 °C. Standard curves were constructed by addition of these solutions of halofantrine hydrochloride and desbutylhalofantrine hydrochloride (10–250 ng; 10–25 μl of the appropriate stock) and the internal standard (100 ng; 10 μl) to drug-free plasma, red blood cells or urine to produce a concentration range of 10–250 μg $l^{-1}$. Single samples, together with appropriate blanks, were run at each concentration. Samples were processed as described above and the peak-height ratio of drug and metabolite was plotted against the corresponding concentration of drug or metabolite. Linear regression of peak-height ratio versus drug or metabolite concentration was performed in order to estimate slope, intercept (peak-height ratio for zero concentration) and correlation coefficient for each standard curve.

*Analytical recovery, assay precision and accuracy*

The analytical recovery of halofantrine and desbutylhalofantrine was estimated by comparison of the peak height obtained from an extracted sample (plasma or red blood cells) containing a known amount of the substance with the peak height obtained from a solution in methanol containing the same amount of each compound. Inter- and intra-assay precision and accuracy were determined by replicate assays of samples from a pool of spiked plasma or red blood cells. The inter-assay variation was assessed over a period of twelve weeks.

*Volunteer study*

One healthy male volunteer aged 31 years, who had been taking no other drugs, received 500 mg (two 250-mg halofantrine hydrochloride tablets) orally after an overnight fast. Venous blood samples (10 ml) were taken pre-dose, then after 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 48, 72 and 168 h. Blood was centrifuged (3000

*g* for 10 min) and the plasma was removed and stored at −70°C until the time of analysis. Urine was collected for 1 h pre-dose (blank) then 0–12, 12–24 and 24–48 h. Urine volume was measured and an aliquot (10 ml) was frozen and stored at −70°C.

*Pharmacokinetic calculations*

The peak plasma concentration of halofantrine and the desbutyl metabolite and the respective times to attain peak plasma concentrations were determined by visual inspection of the log concentration versus time profile. The apparent terminal elimination rate constant ($k$) was calculated by regression analysis of the post-distributive log linear portion of the plasma concentration versus time curve. Apparent terminal elimination half-life of halofantrine, plasma clearance and apparent volume of distribution at equilibrium were calculated using standard, model-independent formulae [6].

## RESULTS AND DISCUSSION

The reported sample preparation was the most rapid and simplest procedure that could be devised to permit adequate extraction from plasma and red blood cells of compounds of interest without interference from endogenous substances and other commonly administered antimalarial agents. For urine samples, it was found that conventional liquid–liquid extractions were sufficient.

Fig. 2 illustrates chromatograms obtained typically from drug-free plasma (a), red blood cells (b) and urine (c), respectively. Fig. 3 illustrates the chromatograms obtained from plasma (a), red blood cells (b) and urine (c) from a healthy human volunteer following the oral administration of halofantrine hydrochloride

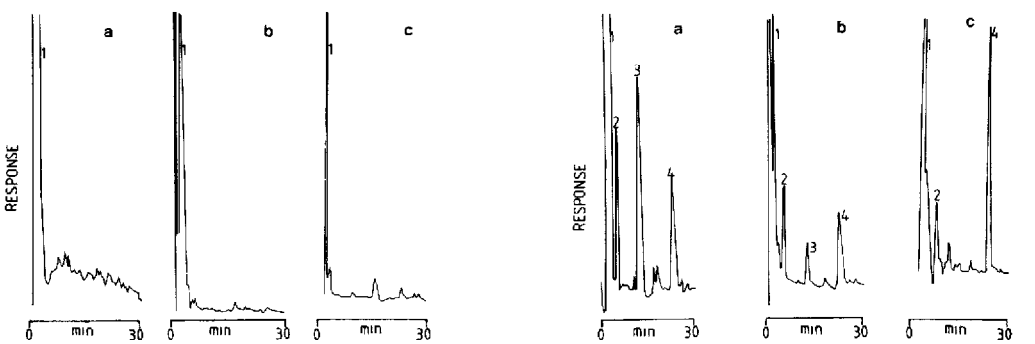


Fig. 2. Typical chromatograms obtained following extraction of drug-free plasma (a), packed red blood cells (b) and urine (c) Attenuation: 0.005 a.u.f.s. Peak 1 = injection event.




Fig. 3. Chromatograms obtained following extraction of plasma (a), packed red blood cells (b) and urine (c) obtained from a healthy human volunteer following oral administration of 500 mg halofantrine hydrochloride. Peaks: 1 = injection event; 2 = desbutylhalofantrine; 3 = halofantrine; 4 = internal standard. Attenuation: 0.005 a.u.f.s. for plasma and red blood cells and 0.002 a.u.f.s. for urine. Estimated concentrations of halofantrine and desbutylhalofantrine (expressed as the hydrochloride salt) were 268 and 34 $\mu g\ l^{-1}$ (plasma) and 148 and 37 $\mu g\ l^{-1}$ (red blood cells) Estimated urinary concentration of desbutylhalofantrine was 11 $\mu g\ l^{-1}$.

(500 mg). Three peaks were resolved to baseline with retention times of 4.3, 10.0 and 22.3 min corresponding to desbutylhalofantrine, halofantrine and the internal standard. Comparison of chromatograms of drug-free plasma, red blood cells or urine with chromatograms from this volunteer study demonstrates that there is no interference from endogenous substances using these methods of sample preparation and chromatography. The relatively rapid elution of desbutylhalofantrine did not produce problems with respect to accurate and precise quantification at low concentrations. In addition, we observed no chromatoraphic interference when aqueous solutions of the commonly used quinoline antimalarials chloroquine, primaquine, amodiaquine, quinine or quinidine were injected into the chromatograph together with halofantrine and the desbutyl metabolite.

Calibration curves (plasma, red blood cells or urine) for both halofantrine and the desbutyl metabolite were linear in the range 0–250 ng ($r \geqslant 0.99$). Analytical recovery from plasma was $98 \pm 5\%$ for halofantrine and $94 \pm 3\%$ for the desbutyl metabolite ($n=6$; 150 $\mu g\, l^{-1}$). The minimum detectable concentration of halofantrine and the desbutyl metabolite (as the hydrochloride salt) corresponding to a peak three times baseline noise at 0.001 a.u.f.s. were 1 and 0.7 $\mu g$, respectively using 1 ml of plasma. For red blood cells, the values were 2 and 1.5 $\mu g\, l^{-1}$ due to a reduction in analytical recovery to $\sim 72\%$ for halofantrine and $\sim 50\%$ for the desbutyl metabolite (50 $\mu g\, l^{-1}$). The stability of halofantrine and the desbutyl metabolite was determined by analysis of samples of plasma or red blood cells containing known concentrations of both compounds stored at $-70\,^{\circ}C$ for up to three months. No significant breakdown of either analyte in plasma or red blood cells was observed over this period. The intra- and inter-assay coefficients of variation (i.e. analytical precision) for plasma were, respectively, 6.1% (45 $\mu g\, l^{-1}$; $n=5$) and 8.7% (30 $\mu g\, l^{-1}$; $n=8$) for halofantrine and 9.6% (45 $\mu g\, l^{-1}$; $n=5$) and 12.3% (30 $\mu g\, l^{-1}$; $n=8$) for the desbutyl metabolite. For red blood cells, the corresponding values were 4.7% (25 $\mu g\, l^{-1}$; $n=5$) and 10.6% (100 $\mu g\, l^{-1}$; $n=5$) for halofantrine and 5.8% (100 $\mu g\, l^{-1}$; $n=5$) and 4.7% (100 $\mu g\, l^{-1}$; $n=6$) for the desbutyl metabolite. Accuracy was assessed as the percentage difference between the actual (i.e. added) concentration and that which was determined analytically. For halofantrine in plasma, the value was 9.3% (45 $\mu g\, l^{-1}$; $n=5$) and for the desbutyl metabolite, 5.3% (45 $\mu g\, l^{-1}$; $n=5$). The corresponding values for red blood cells were 8.8% (25 $\mu g\, l^{-1}$; $n=6$) and 7.8% (100 $\mu g\, l^{-1}$; $n=5$). The urine assay was not fully validated since this was only a minor route of elimination.

The validated method for plasma was used to study the pharmacokinetics of the halofantrine and the desbutyl metabolite in one healthy volunteer after a single oral 500-mg dose of the drug. Plasma concentrations of halofantrine and desbutylhalofantrine were measurable throughout the 168 h of the study. Fig. 4 illustrates plasma concentration (expressed as hydrochloride salt) versus time profiles over the period 0–72 h. Peak plasma concentrations of halofantrine (268 $\mu g\, l^{-1}$) were achieved at 4 h post-dose. Thereafter, concentrations fell monoexponentially with an apparent terminal elimination half-life of 61 h. Desbutylhalofantrine could be detected in plasma 2 h post-dose. Halofantrine was not detected in any urine samples but 0.03% of the dose was excreted as the desbutyl metabolite in urine over 168 h. Plasma clearance and apparent volume of distribution

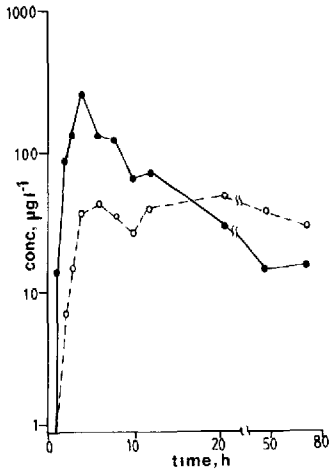


Fig. 4. Plasma concentrations of halofantrine (closed symbols) and desbutylhalofantrine (open symbols) expressed as the hydrochloride salt measured in a healthy human volunteer (0–72 h) following the oral administration of a single dose (500 mg) of halofantrine hydrochloride.

of halofantrine were found to be 137 l $h^{-1}$ and 12 484 l, respectively.

This assay has several advantages over that developed by Hines et al. [5] which has limited sensitivity (50 ng/ml) and is compromised by contaminating endogenous compounds eluting with the void volume. Also, a large volume of blood is required (2.0 ml) when field studies may only permit 0.1–0.5 ml of blood to be taken. Extraction and clean-up procedures are lengthy and complex. The assay described in this report is reproducible, selective and sensitive. Sample preparation is simpler and uses a smaller voume of biological fluid. For these reasons we believe that the improved assay is more suitable for the evaluation of the pharmacokinetics of this increasingly important antimalarial drug after administration of clinically relevant doses to malaria patients under field conditions.

ACKNOWLEDGEMENTS

We are grateful to SKF Research (Welwyn, U.K.) for financial support. K.A.M. is in receipt of a postgraduate studentship from the Research Development Fund of the University of Liverpool.

REFERENCES

1 W. Peters, Br. Med. Bull., 38 (1982) 187.
2 W.T. Colwell, V. Brown, P. Christie, J. Lange, C. Reece, K. Yamamoto and D.W. Henry, J. Med. Chem., 15 (1972) 771.
3 T.M. Cosgriff, E.F. Boudreau, C.L. Pamplin, E.B. Doberstyn, R.E. Desjardins and C.J. Canfield, Am. J. Trop. Med. Hyg., 31 (1980) 1075.
4 J.P. Coulaud, J. Le Bras, S. Matheron, B. Moriniere, A.G. Saimot and J.F Rossignol, Trans. R. Soc. Trop. Med. Hyg., 80 (1986) 615.
5 J.W. Hines, P.D. Elkins, C.E. Cook and C M. Sparacino, J. Pharm. Sci., 74 (1985) 433.
6 M Gibaldi and D. Perrier, in J Swarbrick (Editor), Drugs and the Pharmaceutical Sciences, Vol. I, Marcel Dekker, New York, 1975.