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Short Communication

Measurement of halofantrine and its major metabolite desbutylhalofantrine in plasma and blood by high-performance liquid chromatography: a new methodology

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ABSTRACT

A new high-performance liquid chromatographic assay for the measurement of halofantrine and desbutylhalofantrine in plasma and whole blood is described. The method involves a smaller sample volume, simplified sample pre-treatment and a shorter run-time, and is adaptable to the measurement of samples dried onto filter paper strips. Using this method, which is both selective and sensitive, plasma concentration *versus* time profiles for both substances have been investigated following a single oral dose (500 mg) of halofantrine hydrochloride to a healthy adult volunteer. In addition, a clinical study designed to evaluate the disposition and elimination of the two compounds in children with non-severe falciparum malaria is in progress.

INTRODUCTION

Halofantrine (Hf), as phenanthrene-methanol, is a comparatively recent addition to the drugs used in the treatment of malaria. The determination of Hf and desbutylhalofantrine (Hfm) in biological fluids by reversed-phase high-performance liquid chromatography (HPLC) has been described [1–3], and used in pharmacokinetic studies [4,5]. The reported methods require expensive sample pre-treatment and have a comparatively long run-time (30 min). For pharmacokinetic studies in the tropics there is a need for a simple and reliable method of sample collection and storage, for which absorption of blood or plasma onto filter paper strips is ideal [6]. We have searched for an assay method that would be appropriate for samples dried onto filter paper, and also for improved chromatography.

This paper describes a new assay technique that permits the measurement of Hf and Hfm in liquid and filter paper-absorbed plasma and whole blood. The measurement of drugs in bi-

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ological fluids dried onto filter paper strips has been described for chloroquine [7], quinine and quinidine [6], phenobarbitone [8], pyrimethamine and sulfadoxine [9].

EXPERIMENTAL

Reagents

Halofantrine hydrochloride and desbutylhalofantrine hydrochloride were obtained from Smith Kline Beecham (Welwyn, UK). The internal standard, 2,4-dichloro-9-(2-dibutylamino-1-hydroxy)ethyl-6-trifluoromethylphenanthrene hydrochloride was a gift from Dr.G. Edwards, University of Liverpool (Liverpool, UK). Filter paper strips (Whatman grade 17) were obtained from Whatman (Kent, UK). Acetonitrile and hexane (both HPLC grade), dimethyldichlorosilane, orthophosphoric acid, diethyl ether and ammonia (all AnalaR grade) were obtained from BDH (Poole, UK). Perchloric acid (AnalaR grade) was obtained from E. Merck (Darmstadt, Germany). Stock standard solutions were prepared in ethanol from which working standard solutions were prepared in mobile phase.

Sample handling

Drug-free blood was drawn from volunteers by venepuncture and mixed in heparinized tubes (Sterilin, Feltham, UK). A 2-ml aliquot of blood was transferred to a plastic vial and spiked with known concentrations of Hf and Hfm using a 25-μl glass syringe (SGE, Ringwood, Australia). Then 0.5-ml samples were transferred in duplicate to fresh vials for liquid blood assay, and to filter paper strips using a 1-ml pipette (Gilson pipetman) as described previously [6]. The remaining drug-free blood was centrifuged and the plasma treated similarly. The liquid samples were stored at −80°C in air-tight containers, and the strips were air-dried at 37°C and stored at room temperature protected from dust and direct sunlight. All spiked samples were analysed within a month. Stability experiments indicate that Hf and Hfm measurements (50 and 600 ng/ml) are unaffected by storage of the strips for up to six weeks.

Extraction

Filter paper strips. To the filter paper-absorbed samples, the internal standard (I.S.) (150 ng; 15 μl of a 10 μg/ml aqueous solution) was added using the 25-μl syringe. The strips were then redried at 37°C (0.5 h). The bands of dried blood or plasma were excised, cut into small pieces and transferred to 10-ml silanized glass culture tubes, to which 1 ml of 0.01 *M* perchloric acid in distilled water was added. After vortex-mixing and incubation at room temperature (5 min), 2 ml of acetonitrile were added. After vortex-mixing at high speed for 30 s, 1 ml of ammonia was added and thoroughly mixed. Next, 5 ml of the extracting solvent (hexane) were added and vortex-mixed for 1 min at moderate speed. Following centrifugation (*ca.* 2000 *g* for 5 min) and separation, the organic phase was evaporated to dryness under nitrogen (37°C). Samples were reconstituted in mobile phase (120 μl), and aliquots of 50 μl were injected into the system.

Liquid samples. To the 0.5-ml aliquots of blood or plasma in 10-ml silanized glass culture tubes, the I.S. (150 ng; 15 μl of 10 μg/ml aqueous solution) was added as above. Plasma proteins were precipitated with 2 ml of acetonitrile and vortex-mixed for 30 s, and centrifuged (*ca.* 2000 *g* for 5 min) to sediment the proteins. The supernatant was transferred to a clean silanized glass culture tube, and 0.5 ml of ammonia was added and vortex-mixed. Samples were extracted into hexane–diethyl ether (1:1, v/v; 5.0 ml) by vortex-mixing (1 min). Following centrifugation (*ca.* 2000 *g* for 5 min) and separation, the upper organic phase was evaporated to dryness under nitrogen (37°C). Samples were reconstituted in mobile phase (120 μl), and aliquots of 50 μl were injected into the system.

Chromatography

Chromatography was performed under isocratic conditions using an Isochrom delivery system (model A0099/314, Spectra Physics, San Jose, CA, USA) fitted to a Rheodyne valve injector (equipped with a 50-μl loop) and connected to a stainless steel-column (Hypersil 5 ODS, 25 cm × 4.6 mm I.D., Wellington House, Mac-

clesfield, UK) preceded by a guard column (CN precolumn, RP-18 endcapped 5 μm, 10 mm × 4.6 mm I.D., E. Merck). Column effluent was monitored by a variable-wavelength UV detector (Model A009/307A, Spectra Physics) set at 254 nm. The mobile phase was water–acetonitrile (35:65, v/v) containing triethylamine (1%) and adjusted to pH 4 with orthophosphoric acid. The flow-rate was 2.0 ml/min, generating an operating pressure of *ca.* 140 bar.

Recovery, standard curves and reproducibility

The recoveries of Hf, Hfm, and I.S. were assessed by adding known amounts of these compounds to 1 ml of drug-free blood or plasma as per calibration curve (50–1000 ng/ml). Aliquots of 0.5 ml of blood or plasma were put on separate filter paper strips ($n = 6$), and 150 ng of I.S. were added to each strip before drying and extraction as above. Similarly, six liquid aliquots of 0.5 ml each were put into separate silanized centrifuge tubes, and 150 ng of I.S. were added to each tube before extraction, as described above for liquid samples. The recovery was defined as the ratio of the peak heights for Hf, Hfm and I.S. from the extract to those produced by direct injection of the equivalent amounts. Standard curves were prepared by the addition of Hf and Hfm to drug-free samples, which were extracted with the stored samples in each run. Intra- and inter-assay reproducibilities (coefficient of variation, C.V.) were assessed by the addition of Hf and Hfm to drug-free samples at concentrations of 10, 20, 50, 600 and 1000 ng/ml. Inter-assay reproducibility was assessed weekly over one month using 50 and 600 ng/ml Hf and Hfm ($n = 6$ in all cases).

Volunteer study

One healthy adult male volunteer, who had not been taking other drugs, received a single oral dose of 500 mg of Hf · HCl. Venous blood samples (5 ml) were taken pre-dose, then after 1, 2, 3, 4, 5, 6, 8, 10, 24, 36, 52, 72 and 96 h. Blood was centrifuged (*ca.* 2000 *g* for 5 min) and the plasma removed, and 0.5-ml aliquots were dried onto filter paper strips. The strips were stored at room temperature and the liquid samples at −80°C until assay.

Data analysis

The peak plasma concentrations of Hf and Hfm and the respective times to attain peak plasma concentrations were determined by visual inspection of the log concentration *versus* time profiles. Pharmacokinetic parameters were determined using model-independent formulae [10]. The terminal elimination rate constant (K_{el}) was calculated by regression analysis of the post-distributive log-linear portion of the plasma concentration *versus* time curves. The area under the curve (AUC_{0-t}) was determined by the trapezoidal rule [11].

RESULTS

Chromatography

Hfm, Hf and I.S. were resolved to baseline over the concentration range studied (10–2000

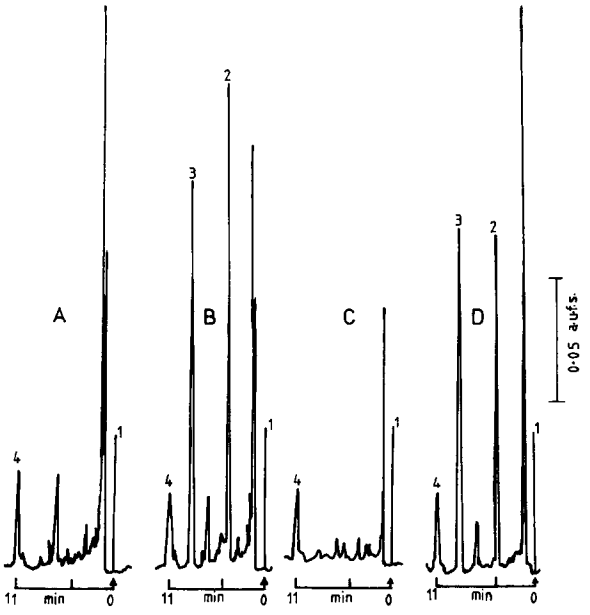

Fig. 1. Chromatograms obtained following extraction of 0.5 ml of drug-free liquid plasma (A), 0.5 ml of liquid plasma containing 1 μg/ml Hf and Hfm (B), 0.5 ml of drug-free plasma dried on a filter paper strip (C), and 0.5 ml of plasma containing 1 μg/ml Hf and Hfm dried on a filter paper strip (D). Peaks: 1 = injection; 2 = desbutylhalofantrine; 3 = halofantrine; 4 = internal standard. Comparable chromatograms were obtained following extraction from liquid and filter paper-absorbed whole blood samples.

TABLE I

RECOVERY, REPRODUCIBILITY AND STANDARD CURVE CORRELATION

Data obtained from spiked whole blood and plasma liquid and filter paper-absorbed whole blood and plasma samples.

Matrix	Compound	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)		Correlation coefficient (r^2)
			Intra-assay	Inter-assay	
Liquid samples					
Plasma	Hf	96 ± 12	4.4	8.4	0.9942
	Hfm	97 ± 9	6.0	8.7	0.9945
Whole blood	Hf	90 ± 11	6.2	9.4	0.9971
	Hfm	87 ± 12	8.0	10.4	0.9850
Filter paper-absorbed samples					
Plasma	Hf	68 ± 8	7.2	7.9	0.9866
	Hfm	67 ± 4	6.2	9.3	0.9938
Whole blood	Hf	65 ± 6	8.4	9.7	0.9850
	Hfm	63 ± 5	10.3	10.7	0.9893

ng/ml) with retention times of 4.5, 8.5 and 11.0 min, respectively (Fig. 1). There was no interference from quinine, quinidine, chloroquine, phenobarbitone, sulfadoxine, pyrimethamine, proguanil, cycloguanil, chlorproguanil, paracetamol, mefloquine and chlorcyclogunail, none being detected on chromatography, or diazepam, which was eluted at 3 min. The lowest measurable concentration of both Hf and Hfm from a 0.5-ml liquid or filter paper-absorbed sample was 10 ng/ml, which at 0.02 a.u.f.s. consistently produced a peak at least four times the background noise.

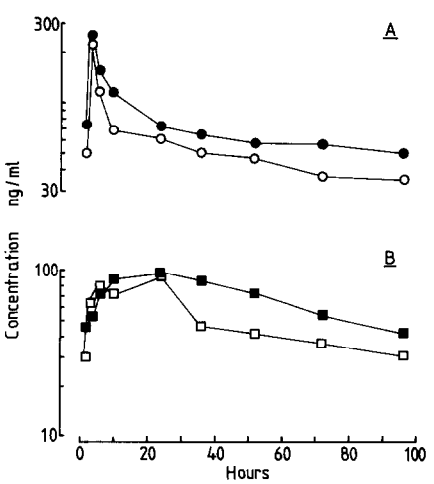

Fig. 2. Plasma concentrations of Hf and Hfm measured in a healthy volunteer (0–96 h) following oral administration of a single dose (500 mg) of Hf hydrochloride. (A) Hf concentrations in liquid (●) and in filter paper-absorbed (○) plasma samples. (B) Hfm concentrations in liquid (■) and in filter paper-absorbed (□) plasma samples.

Recovery, standard curves and reproducibility

Assay, recoveries, correlation coefficients (r^2) and reproducibilities for Hf and Hfm are shown in Table I. The recovery of I.S. was 90 ± 19% in blood and plasma samples, and 68 ± 5% in filter paper-absorbed blood and plasma samples (n = 5).

TABLE II

PHARMACOKINETIC PARAMETERS

Results obtained after oral administration of Hf hydrochloride (500 mg) to one healthy adult volunteer. A, Liquid plasma samples; B, filter paper-absorbed plasma samples.

Compound	Sample	C_{max} (ng/ml)	t_{max} (h)	AUC (mg/h/l)
Hf	A	252	4	6.830
	B	228	4	5.174
Hfm	A	95	24	6.581
	B	90	24	4.757

Volunteer data

Concentrations of Hf and Hfm were measurable in both liquid plasma and plasma dried onto strips throughout the 96 h of the study. Fig. 2 illustrates plasma concentration *versus* time profiles for both sampling methods. Hfm was detected in plasma 2 h after the dose. The pharmacokinetic parameters for Hf and Hfm derived from the volunteer, summarized in Table II, are comparable with those reported elsewhere [2,4].

DISCUSSION

Our assay and its adaptation to filter paper-absorbed samples facilitates the collection, transportation, storage and estimation of Hf and Hfm with high selectivity. The sensitivity (10 ng/ml), although lower than those of other methods [2,3] is suitable for the measurement of concentrations in the therapeutic range. This assay has several advantages in comparison with previous methods. It requires a smaller sample volume (0.5 ml), which is important in clinical studies in young children. The run-time has been reduced from 22–30 min to 12 min. The extraction time has also been reduced because the method avoids the multiple centrifugation associated with sample pre-treatment cartridges. The extraction solvents are lighter than the aqueous phase. In addition to these advantages, the filter paper-absorbed samples produce chromatograms with less interference from endogenous blood components without the need for a deproteinization stage, which further reduces the extraction time. Hexane–diethyl ether gives the highest recovery from liquid samples, whereas hexane alone produces chromatograms with less interference from endogenous compounds in filter paper-absorbed samples. The method is reproducible and suitable for evaluation of the clinical pharmacokinetics of Hf and Hfm in patients being treated for malaria under field conditions. Although the recoveries of Hf and Hfm from filter paper-absorbed samples were less than those from liquid samples, this did not affect the lowest measurable concentration, which remained 10 ng/ml in both cases. The advantages of the collection of blood or plasma samples onto filter paper strips for drug measurement have been described in full elsewhere [6], and may be applicable to other drugs in the tropics. Although the I.S. used in our study is not readily available, diazepam would be an appropriate alternative.

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