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New solid-phase extraction for an improved high-performance liquid chromatographic procedure for the quantitation of halofantrine and monodesbutylhalofantrine in blood or plasma

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Abstract

A rapid, accurate, and sensitive high-performance liquid chromatographic (HPLC) method, with fluorimetric detection, for the simultaneous measurement of halofantrine and desbutylhalofantrine in human plasma or whole blood is described. Sample preparation involved protein precipitation, followed by an efficient solid-phase extraction on a C_8 cartridge. Analytes were isolated from 1 ml of the biological fluids and recovered by a 2% acetic acid in ethyl acetate solution. Chromatographic separation was carried out on a LiChrospher 60 RP select B, C_8 bonded phase (5 μm particle size, 25 cm × 4 mm I.D.) using a mobile phase of water–acetonitrile (35:65, v/v) containing triethylamine (1%) and adjusted to pH 4 with orthophosphoric acid. The total run time was 14 min. Relative standard deviations of the intra- and inter-assay precisions were less than 5.9%. Assumption of linearity was investigated by studying the *y*-residuals and by ANOVA (analysis of variance). Because of the wide range of calibration (0.1 to 2.0 μg/ml) variances were non-homogeneous (Hartley's test) and the weighted regression line was computed in order to allow pharmacokinetic studies. Accuracy was tested using a *t*-statistic. Limits of decision, detection and quantification were realized from an analysis of the blanks. Application of the method to clinical specimens was demonstrated.

1. Introduction

Efforts to develop new anti-malarial drugs have shown that some phenanthrene-methanols may be effective agents. Halofantrine (HAL) (Fig. 1) synthesized originally by Colwell et al.

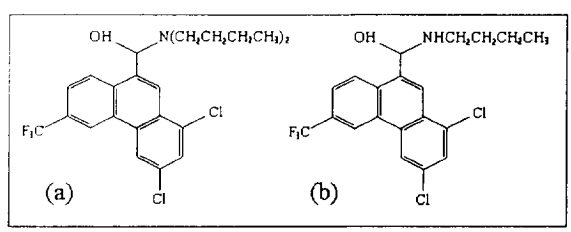

Fig. 1. Chemical structure of (a) halofantrine and (b) monodesbutylhalofantrine.

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[1] appears to be effective in vivo against multidrug resistant *Plasmodium falciparum* [2–4]. In areas where the parasite was known to be chloroquine-resistant, HAL represents with quinine the treatment of choice of malaria. Unfortunately, the currently available formulation is poorly and erratically absorbed from the gastrointestinal tract as a result of its limited solubility [5–7]. Moreover, the drug has recently proved to present cardiotoxic effects [6]. Thus, in order that the drug may be used optimally in malaria treatment, a detailed study of the clinical pharmacology of HAL is required. This demands that suitable sensitive and selective analytical methods are available. Hines et al. [9] have presented the first high-performance liquid chromatographic procedure for the analysis of HAL in whole blood. Gawienowski et al. [10] and Milton et al. [11] have reported HPLC methods for the simultaneous measurement of HAL and its active metabolite monodesbutylhalofantrine (MDBH) in human plasma, which are both sensitive. Disadvantages of the two methods, however, include a lengthy analytical run time of about 23 min. Two recent papers [12,13] reported a more rapid HPLC assay: the first assay technique allowed the measurement of the drugs in plasma and whole blood on filter paper strips, whereas the other technique used solid-phase extraction (SPE) for sample clean-up followed by liquid–liquid extraction.

On the other hand, since HAL and its active metabolite contain a chiral centre, chiral separation of HAL [14] and direct determination of the enantiomers of both drugs [15] have been reported. Gimenez et al. [16] have more recently studied the plasma concentrations of the enantiomers of HAL and MDBH in malaria patients.

From all these methods, the technique of Keeratithakul et al. [13] seems to be the most interesting. However, the multi-step extraction, performed according to the method of Milton et al. [11] is tedious and time-consuming. That is the reason why we have developed a new solid-phase extraction which permits in a single step an efficient, rapid and simple extraction of HAL and MDBH in plasma and whole blood. Assay sensitivity was increased by using fluorescence rather than UV detection.

2. Experimental

2.1. Materials

The solid-phase extraction was performed using Vac Elut SPS 24 (Analytichem International, Paris, France) and Bond Elut C_8 (200 mg/3 ml) (Interchim, Montluçon, France) extraction cartridges.

The HPLC system consisted of a Waters 717 autosampler (Milford, MA, USA) and a quaternary low-pressure pump Waters 590, which were connected to a Waters 470 scanning fluorescence detector. Signal was recorded by the Maxima 820 software from Waters. Chromatographic separation was achieved at ambient temperature on a Lichrospher 60 RP select B C_8 bonded phase (5 μm particle size, 25 cm × 4 mm I.D.) from Hewlett-Packard (Les Ulis, France), while the chromatographic run time was 14 min. The fluorescence detector settings were: 300 nm excitation wavelength and 375 nm emission wavelength.

2.2. Chemicals

Halofantrine hydrochloride was a gift from SK&F Research (Wellwyn, UK). Desbutylhalofantrine hydrochloride was kindly supplied by the Walter Reed Army Institute of Research (Washington, DC, USA). Internal standard (I.S.), N-*tert.*-butyl-3-hydroxy(1,3-dichloro-6-trifluoromethyl-9-phenanthryl)propionamide hydrate was obtained from Aldrich (ref.: S76,395-0) (Saint Quentin Fallavier, France). All solvents were of HPLC grade: acetonitrile was from BDH (Poole, UK) and acetic acid, orthophosphoric acid, triethylamine and ethyl acetate were from Merck (Darmstadt, Germany).

The mobile phase was water–acetonitrile (35:65, v/v) containing triethylamine (1%) and adjusted to pH = 4 with orthophosphoric acid. Flow-rate was set at 1.1 ml/min.

2.3. Standards

Stock solutions (1 mg base/ml) of HAL and MDBH were prepared in acetonitrile–water

(50:50, v/v). A 200-μl volume of the HAL and MDBH stock solutions were evaporated to dryness and reconstituted by addition of 100 ml of drug-free plasma. Other points in the calibration range were obtained by diluting this standard solution 1:1, 1:9, and 1:19 with drug-free plasma. The solutions were stored in 2-ml vials (Eppendorf, from ATGC, Paris, France) at −30°C until assay.

An internal standard working solution (10 μg/ml) was prepared by dissolving 10 mg of the drug in 1000 ml of acetonitrile–water (50:50, v/v), this was stored at 4°C.

The bicarbonate buffer for solid-phase extraction was prepared by dissolving 1 g of potassium bicarbonate ($KHCO_3$, Merck) in 100 ml of water and was stored at 4°C.

2.4. Solid-phase extraction

Plasma samples

To 1 ml of blank plasma, standard calibration or plasma sample were added 100 μl of working internal standard solution and 2 ml of acetonitrile to precipitate proteins. After vortex-mixing for 15 s, all samples were centrifuged at 1500 *g* for 10 min. The supernatant was then transferred to the extraction cartridges which were successively conditioned twice with 2 ml methanol and twice with 2 ml bicarbonate buffer. Pretreated plasma samples or standards were allowed to drain through under vacuum and left to dry for 1 min.

Extraction cartridges were washed twice with 2 ml bicarbonate buffer and twice with 2 ml of methanol–water (50:50, v/v). The analytes were eluted with four volumes of 750 μl of 2% acetic acid in ethyl acetate. The eluate was evaporated to dryness under a stream of nitrogen at 40°C and redissolved in 200 μl of the mobile phase; 50 μl were injected onto the analytical column.

Whole blood

Blood samples or standards were freezed first, then defrosted, diluted 1:1 with distilled water and centrifuged at 5000 *g* for 5 min. An aliquot of 1 ml of the supernatant (diluted to 1:1) was then treated and extracted under the same conditions as for a plasma sample. After evaporation of the eluent, the residue was reconstituted in 100 μl of mobile phase and 50 μl were injected.

3. Method validation

3.1. Within- and between-day precisions

The within-run precision was calculated from repeated analyses during one working day. The day-to-day precision was obtained from repeated analyses of standards calibration on ten successive working days.

3.2. Recovery

The recovery of an analyte is calculated by comparing peak-area ratios of the analyte to internal standard with and without extraction. In both cases the internal standard is added just before injection.

3.3. Linearity

The equation of the least-squares line is calculated, then the quality of the linear model is carefully investigated as follows:

(1) The first approach is to perform a study of the *y*-residuals, which is a simple and instructive test of whether a linear plot is appropriate [17–21]: from the equation of the linear regression, we can calculate the $y - y_{cal}$-values which represent the differences between the experimental *y*-values and the fitted *y*-values. The residuals thus represent the random experimental errors in the measurement of *y*, if the statistical model used (the unweighted regression line of *y* on *x*) is correct. The residuals are assumed to be normally distributed.

(2) Then, a more sophisticated test to assume linearity can be applied: analysis of variance (ANOVA) using the *F*-test [19,20]. ANOVA is a powerful and very general method which separates the contributions to the overall variation in a set of experimental data and tests their significance. There are two contributions: one due to regression, and one not described by the linear model, i.e. residual.

3.4. Choice of the model: weighted or unweighted

After affirmation of linearity, the model of the linear regression must be chosen. In a wide dynamic range of calibration the y-errors tend to increase as x increases, i.e. non-homogeneity of the variances.

Hartley's test (r) can be used to easily decide on the necessity of a weighted linear regression in biomedical analysis. The calculated r-value is the ratio between the highest and the lowest variance in the range of k points of calibration. If the variances are non-homogeneous, the regression line must be a weighted one. The equation for this weighted line differs from classical equations because a weighting factor, ω_i, must be associated with each calibration point x_i, y_i. This factor is inversely proportional to the variance of y_i, s_i^2.

Calculations of the weighted regression lines are evidently more complex than an unweighted regression computation. However, we must encourage such computation since this model is the only one that gives the proper estimates of standard deviations and confidence limits when the weights vary significantly with x_i. It must be used especially in pharmacokinetic studies [18–20].

3.5. Accuracy

The mean measured value is compared to a point (true value) using a t-statistic. If the null hypothesis is accepted, the measured and the true values are not different [22]. In our study, we have tested accuracy at the lowest and the highest point of the calibration range.

3.6. Assay detection limits

Limit of decision: L_c

The critical value, L_c, thus depends on both the standard deviation of the distribution and the risk one is willing to take of making a wrong decision. The decision limit can be expressed in terms of signals by: $L_c = m_{bl} + K_c s_{bl}$ (m_{bl} = mean of blanks, s_{bl} = standard deviation of the blanks for $n = 30$).

Introducing a value of $K_c = 3$ leads to a probability $1 - \alpha = 99.87\%$ if y_{bl} is normally distributed. The error of the first type (deciding that the component is present when it is not) is small ($\alpha = 0.13\%$), whereas the error of the second type (deciding that the component is absent when it is present) is large ($\beta = 50\%$). Signals larger than L_c can be interpreted as the detection of the compound with great certainty, whereas signals smaller than L_c can be interpreted as the absence of the component with poor certainty ($< 50\%$).

Limit of detection: L_d

The limit of detection should be defined in such way that α and β are well balanced [23–25]. $L_d = m_{bl} + K_d s_{bl}$; if a concentration is equal to the detection limit, for which $K_d = 6$, it can be detected with 99.87% certainty. Smaller concentrations can be detected with less confidence.

Limit of quantification: L_q

A determination or quantification limit can be defined as the limit at which a given procedure will be sufficiently precise to yield a satisfactory quantitative estimate of the unknown concentration. The limit of quantification is the concentration that can be determined with a fixed minimum relative standard deviation. Such a limit, L_q, can be determined in terms of m_{bl} and s_{bl}, again assuming that the standard deviations for blank and unknown are identical.

$L_q = m_{bl} + K_q s_{bl}$, K_q is 20 if the maximum allowed relative standard deviation is 5%, K_q is 10 if the relative standard deviation is 10%.

3.7. Interferences

Retention times of other anti-malarial compounds were investigated in order to check their interference with the method. This included quinine, chloroquine, proguanil, pyrimethamine, mefloquine, sulfadoxine, and their major metabolites. Interferences with endogenous compounds were also investigated (Fig. 2a).

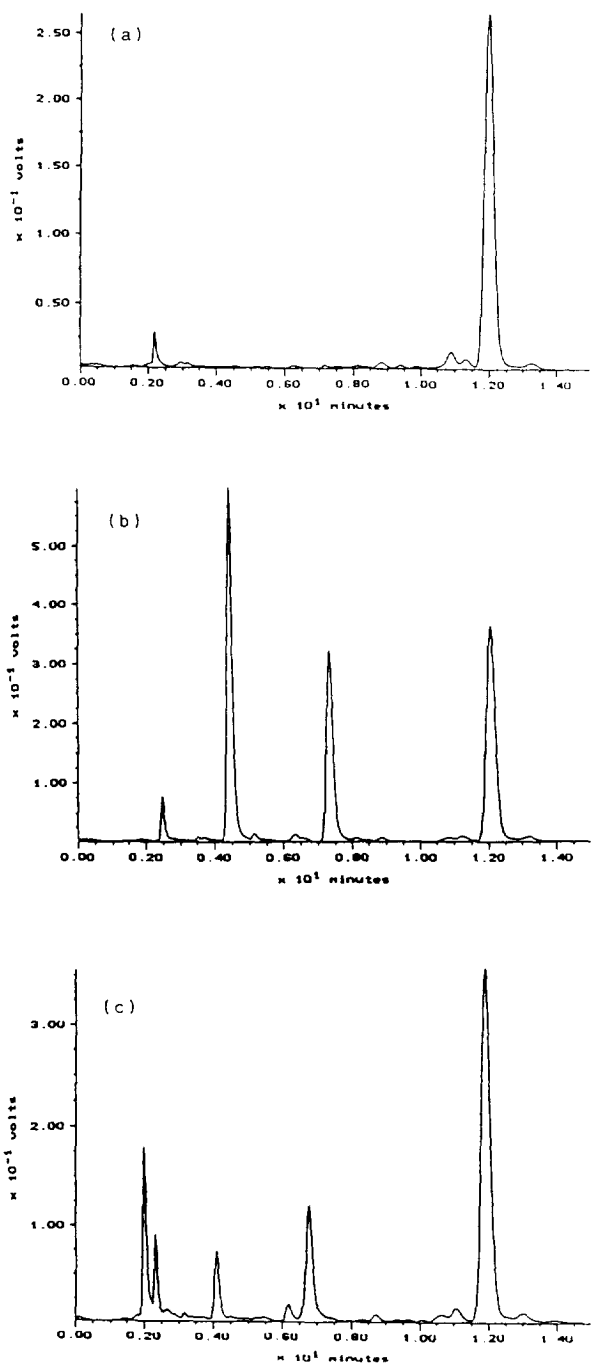

Fig. 2. Chromatograms of (a) blank plasma, (b) standard plasma spiked with 1 μg/ml of MDBH and HAL and (c) plasma sample obtained from a patient with uncomplicated falciparum malaria, following halofantrine treatment. Peaks: 4.1 min = MDBH (120 ng/ml), 6.8 min = HAL (365 ng/ml), 12.0 min = I.S.

4. Results and discussion

4.1. Chromatographic separation

Retention times of MDBH, HAL and the internal standard were 4.1, 6.8 and 12.0 min, respectively. Relative retention times (RRTs), were 0.31 for MDBH and 0.48 for HAL. Using the described conditions, typical chromatograms of MDBH, HAL and I.S. are shown in Fig. 2. The chromatograms illustrate (a) a drug-free plasma, (b) a spiked plasma sample and (c) a plasma sample obtained from a patient with uncomplicated falciparum malaria, following halofantrine treatment.

4.2. Precision

A between-day precision study is summarized in Table 1. The R.S.D.s (%) of the within-day precision were always less than 4.6% ($n = 6$, for each level of the range of calibration).

4.3. Recovery

Extraction recoveries for 200 and 1000 μg/l MDBH ($n = 10$) were respectively 84.4% and 87.7% in plasma; and 81.3% and 78.5% in whole blood. Recoveries for 200 and 1000 μg/l HAL ($n = 10$) were respectively 76.4% and 76.5% in plasma; and 86.4% and 80.5% in whole blood.

4.4. Assumption of linearity

Least-squares regression lines

The following equations of least-squares lines were calculated: (i) plasma: MDBH, $y = 0.0008406x + 0.0073768$ ($r = 0.9995$); HAL, $y = 0.0006131x + 0.0095847$ ($r = 0.9998$); (ii) blood: MDBH, $y = 0.0008348x - 0.0163438$ ($r = 0.9987$); HAL, $y = 0.0007068x - 0.0080833$ ($r = 0.9989$).

Residuals studies

For each drug graphs of the residuals were plotted. Residuals are normally distributed but

Table 1
Between-day precision study

Concentration	Plasma		Whole blood	
	Peak-area ratio analyte/I.S. (mean ± S.D.)	R.S.D. (%)	Peak-area ratio analyte/I.S. (mean ± S.D.)	R.S.D. (%)
MDBH				
100	0.085634 ± 0.001502	1.8	0.076634 ± 0.004510	5.9
200	0.168265 ± 0.002748	1.6	0.161997 ± 0.003119	1.9
1000	0.872015 ± 0.026187	3.0	0.779981 ± 0.036824	4.7
2000	1.677565 ± 0.025518	1.5	1.670970 ± 0.041112	2.5
HAL				
100	0.067400 ± 0.001481	2.2	0.066159 ± 0.002825	4.3
200	0.128900 ± 0.002468	1.9	0.145777 ± 0.006376	4.4
1000	0.635400 ± 0.016401	2.6	0.669569 ± 0.021197	3.2
2000	1.230000 ± 0.016393	1.3	1.418859 ± 0.034424	2.4

tend to become larger as x increases. A weighted regression line seems to be indicated.

Analysis of variance

In case of MDBH in plasma: $MS^2_{\text{regression}} = 16.4462$ and $MS^2_{\text{residual}} = 0.00249$, where MS = mean square; $F_{\text{cal}} = MS^2_{\text{regression}}/MS^2_{\text{residual}} = 6604.9 \gg F_{\text{the}}$; F_{the} is for $(1, n-2)$DF (F_{cal} 1,8 = 5.32) (DF = degree of freedom) so, the source of variation is well described by the regression, and the model (linear regression) can be considered as correct. Validation of linearity was done the same way for each analyte in both biological fluids. F_{cal} was always found to be more than 479.

4.5. Choice of the model: weighted or unweighted

Hartley's test

In case of MDBH in plasma: $r_{\text{cal}} = s^2_{\max}/s^2_{\min} = 17.43$; $r_{\text{table}}\ (\text{k},\nu) = r_{\text{table}}\ (4,9) = 6.31$, where $r_{\text{table}}\ (\text{k},\nu)$ is r given in a table for k (number of calibration points) and $\nu = n - 1$ degrees of freedom. $r_{\text{cal}} > r_{\text{table}}$, thus variances are non-homogeneous, linear regression must be a weighted one; r_{cal} was always found to be more than 9.11.

Weighted linear equations

Plasma: MDBH, $y_\omega = 0.0008440x_\omega + 0.0011671$; HAL, $y_\omega = 0.0006151x_\omega + 0.0063073$. Blood: MDBH, $y_\omega = 0.0008280x_\omega - 0.0058335$; HAL, $y_\omega = 0.0007008x_\omega - 0.0025201$.

4.6. Accuracy

t_{obs} for each drug was always found to be inferior to 1.82; t_{table} ($\alpha = 5\%$, $n - 1$ DF) = 2.26. With these conditions the hypothesis of equality must be accepted for the two concentrations tested.

4.7. Assay detection limits

Limits of decision (L_c), detection (L_d) and quantification (L_q) for MDBH were respectively 2.4, 5.0, 8.6 μg/l in plasma and 23.4, 32.6, 44.9 μg/l in whole blood. L_c, L_d and L_q for HAL were respectively 1.4, 6.1, 12.4 μg/l in plasma and 7.8, 10.8, 14.8 μg/l in whole blood.

4.8. Interferences

Retention times of other anti-malarial drugs were investigated in order to check their interference with our method. Normal serum compo-

nents or tested drugs did not interfere with the analysis of MDBH and HAL.

5. Conclusion

The chromatographic conditions described here represent an improved modification of the HPLC procedure of Gawienowski et al. [10]. Solid-phase extraction of the last published method [11] was simplified: the additional liquid–liquid extraction step was eliminated by use of a selective elution solvent (2% acetic acid in ethyl acetate). Chromatograms were appreciably cleaner.

This HPLC assay for simultaneous measurement of HAL and MDBH is simple, accurate, and specific. The method has been validated for both therapeutic drug monitoring and pharmacokinetic studies.

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