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DETERMINATION OF MEFLOQUINE BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY AFTER PHOSGENE DERIVATIZATION IN BIOLOGICAL SAMPLES AND IN CAPILLARY BLOOD COLLECTED ON FILTER PAPER

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SUMMARY

Mefloquine is determined in 100-μl samples of whole blood, plasma and capillary blood collected on filter paper by gas chromatography with electron-capture detection after derivatization with phosgene. Sample preparation for whole blood and plasma involves a protein precipitation step that uses a combination of zinc and acetonitrile, followed by simultaneous extraction with methylene chloride and derivatization with phosgene at pH 9.50. Filter paper spots are immersed for 12–24 h in 0.1 *M* hydrochloric acid, followed by simultaneous extraction with methyl *tert.*-butyl ether and derivatization. After evaporation of the organic phase and reconstitution with ethyl acetate, 1 μl of the extract is injected into a megabore capillary column. Because of the high sensitivity of the method, mefloquine concentrations down to 25 nmol/l (9.5 μg/l) are determined in 100-μl samples with a relative standard deviation of 12% at the 25 nmol/l level. Excellent precision was obtained over the range of concentrations tested, 0.10–3 μmol/l (45–1100 μg/l), in both plasma and whole blood and from filter-paper-collected capillary blood. The day-to-day relative standard deviation in plasma at the therapeutic level (1–3 μmol/l) was 4.5% ($n=8$).

INTRODUCTION

Mefloquine (MQ), D,L-*erythro*-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol (Fig. 1), is a new drug effective against multiple-drug-resistant malaria. Its therapeutic value in clinical malaria has been described [1].

Several gas chromatograhic (GC) methods have been reported [2–5] for determining MQ in biological samples, which used silyl [2–4], perfluoroacyl [5], or

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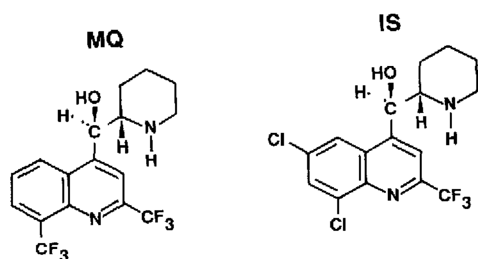

Fig. 1. Structures of mefloquine (MQ) and the internal standard (IS).

silyl and perfluoroacyl [3] derivatization procedures and either electron-capture detection (ECD) or mass spectrometric detection. The detection limits for the methods described in refs. 2 and 3 are between 2.6 and 26 nmol/l (1–10 μg/l) using sample sizes in the range 0.1–1.0 ml. High-performance liquid chromatographic (HPLC) assays have been reported for determination of MQ [6,7] and for simultaneous determination of MQ and the carboxylic metabolite [8–10]. The limit of detection for the method described in ref. 10 is 100 nmol/l for MQ and metabolite using a 0.25-ml sample.

A method with high sensitivity is required to quantify MQ in 50–100 μl sample volumes of capillary blood collected on filter paper, as has been done in the past for assaying other antimalarial drugs, such as chloroquine and its major metabolite [11, 12] and sulfadoxine [13]. Formation of an MQ derivative with good stability and GC properties permits sensitive GC–ECD quantification of MQ and an appropriate internal standard (I.S.), D,L-*erythro*-α-(2-piperidyl)-2-trifluoromethyl-6,8-dichloro-4-quinolinemethanol (Fig. 1), both of which have strong electron affinities owing to the aromatic perfluoromethyl groups. Carbonic dichloride (phosgene) has been recently shown to react readily with MQ under mild conditions to form a cyclic carbonate structure that eliminates the polar hydroxy and secondary aliphatic amine nature in MQ [14]. Phosgene is used in the present work to produce derivatives of both MQ and the analogue I.S., which have excellent GC properties. The resulting GC–ECD method rapidly, sensitively and selectively assays MQ in whole blood and plasma and also permits application to analysis of capillary blood collected on filter paper.

EXPERIMENTAL*

Chemicals

Phosgene (2 mol/l in toluene) was obtained from Fluka (Buchs, Switzerland) and methyl *tert.*-butyl ether (MTBE), methylene chloride and ethyl acetate were from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Mefloquine was kindly supplied by Roche-Produkter (Skarholmen, Sweden) and the I.S. from Walter Reed Army Institute of Research (Washington, DC, U.S.A.). All other chemicals used were of analytical-reagent grade. Stock solution at a concentration of 500

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μmol/l MQ was dissolved in 10 mmol/l hydrochloric acid, and the appropriate working solution (200 μmol/l) was freshly prepared each week by dilution of the stock solution with deionized water. This solution (MQ) was used for plasma or whole blood spiking. To test the accuracy of the dilution and the stability of stock and working standard solutions, molar absorptivities (a) were measured periodically. For MQ $a = 5400 \pm 67$ cm^{-1} M^{-1} at 290 nm in 4 mmol/l hydrochloric acid.

Gas chromatography

A Hewlett-Packard Model 5880 gas chromatograph equipped with a nickel-63 electron-capture detector and a Model 7672A autosampler was used. The detector was operated at 300°C. A bonded-phase megabore column from SGE (Australia), 20 m×0.53 mm I.D., coated with BP10 as stationary phase to a film thickness of 1.0 μm, was operated at 265°C with nitrogen as carrier gas. The flow-rate was 4 ml/min. Injection was on-column.

Collection of volunteer blood samples

Whole blood was drawn by venipuncture into heparinized tubes from one volunteer undergoing a chemoprophylaxic regimen of MQ (250 mg per week). The blood was taken during the second week of prophylaxis at four different times. Corresponding filter-paper-collected capillary blood was taken at the same times using 100-μl heparinized capillary tubes. The filter papers were stored at +4°C for ca. 30 days before analysis.

Whole blood and plasma samples were taken from subjects undergoing mefloquine prophylaxis (250 mg per week). These samples were used to examine the correlation between the present GC method and an HPLC method [10].

Stability of MQ in blood spots on filter paper

To evaluate the storage stability of MQ on filter paper, whole blood was spiked with MQ to a concentration of ca. 3.25 μmol/l. Multiple applications (100 μl per spot) of the spiked whole blood were spotted onto filter paper (Whatman 17Chr). These spots were analysed in duplicate immediately after spotting, and 3, 10, 17, and 20 days after spotting. Samples were stored at room temperature (20–23°C) or at +4°C. The concentration of MQ in the spots was calculated relative to corresponding frozen whole blood standards assayed in the same run.

Assay methods

Whole blood and plasma. To 100-μl samples of whole blood, plasma or spiked standards, 100 μl of I.S. (10 μmol/l) were added in polypropylene tubes. The samples were deproteinized by the addition of 20 μl of 0.2 M zinc sulphate, followed by 200 μl of acetonitrile. The solution was thoroughly vortex-mixed after the addition of each, and the mixture was allowed to stand at room temperature for 15 min. The tubes were centrifuged at 13 400 g for 6 min and the clear supernatant was transferred to glass tubes. Then 3 ml of 0.4 M carbonate buffer (pH 9.5) and 6 ml of methylene chloride, containing phosgene at a concentration equivalent to 10 μl of phosgene (2 mol/l) per 6 ml of methylene chloride, were added. The solution was rotated for extraction for 15 min. After phase separation

the organic phase was evaporated to dryness, and the residue was reconstituted in 300 μl of ethyl acetate or, at the low concentration levels, 100 μl). A 1-μl amount was injected into the gas chromatograph.

Filter paper spots. Filter papers containing dried capillary blood (100 μl) spots were cut into three or four pieces and transferred to a glass tube. To each glass tube were added 100 μl of I.S. (10 μmol/l) and 2 ml of 0.1 *M* hydrochloric acid. Vortex-mixing followed, and the spots were allowed to stand overnight (15–24 h) before ultrasonication for 10 min. A 3.0-ml amount of 0.4 *M* carbonate buffer (pH 9.5) and 6 ml of MTBE containing phosgene at a concentration equivalent to 10 μl of phosgene (2 mol/l) per 6 ml of MTBE were added. The extraction was carried out with the cut-up filter paper in the glass tube. The glass tubes were centrifuged at ca. 3000 *g* for 5 min; the organic phase was transferred to a clean glass tube, evaporated to dryness and reconstituted in 300 μl of ethyl acetate. A 1-μl amount of ethyl acetate was injected into the gas chromatograph.

Recovery

The recovery of MQ was measured by adding known amounts of MQ to plasma, whole blood and whole blood spotted on filter paper. Extraction yields from plasma, whole blood and blood spots on filter paper were calculated relative to those found from extraction of MQ from demineralized water.

Sample processing

To evaluate the filter paper method, blood samples from venipuncture samples were spotted (100 μl) on Whatman chromatography paper 17 Chr (Whatman, Maidstone, U.K.). The filter papers were stored at +4°C until assayed.

RESULTS AND DISCUSSION

The cyclization reaction

The MQ piperidyl side-chain and the benzylic hydroxyl group react with phosgene as described in ref. 14 and outlined in Fig. 2. The derivatization reaction is the same for MQ and for I.S. Fig. 3 shows an HPLC profile of a mixture of MQ and I.S. before and after derivatization. The mobile phase was 0.1 *M* phosphate buffer–acetonitrile (50:50), pH adjusted to 2.50, with Spherisorb ODSI as the stationary phase. As indicated in this figure, each compound is converted into a

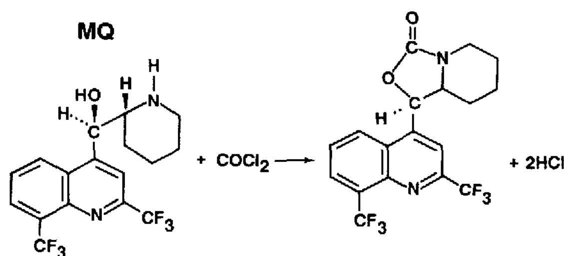

Fig. 2. Cyclization of mefloquine (MQ) between the piperidyl and methanol group with phosgene.

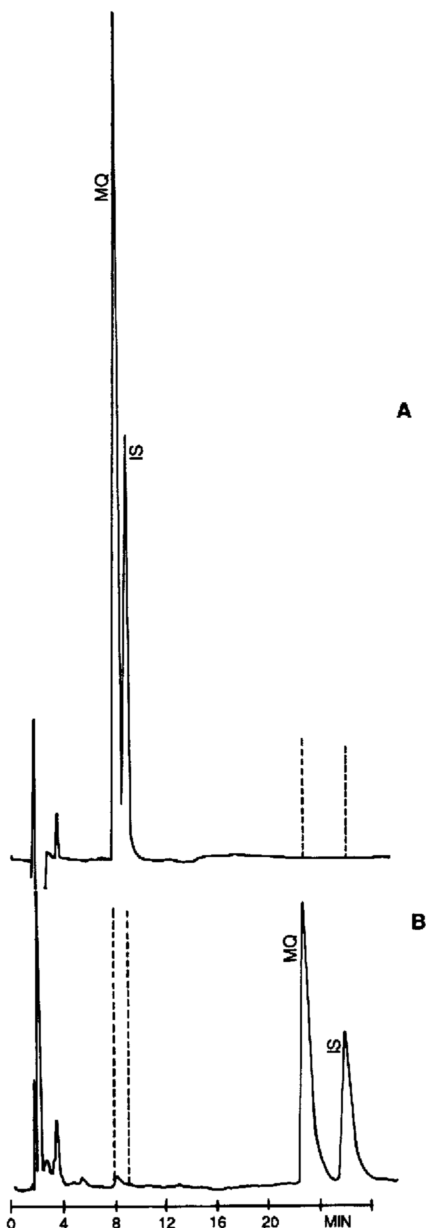

Fig. 3. High-performance liquid chromatograms of mefloquine (MQ) and internal standard (IS). (A) Before phosgene derivatization; (B) after phosgene derivatization.

corresponding structure that is more hydrophobic. The derivatization products of MQ and I.S. were stable for at least one week at 50°C.

The phosgene derivatization technique for 2-aminoalcohols has been studied by Gyllenhaal and Vessman [15,16], and the optimum conditions for the derivatization were derived by a series of pilot experiments using these reports as a guide. Methylene chloride was used as extraction and derivatization solvent for

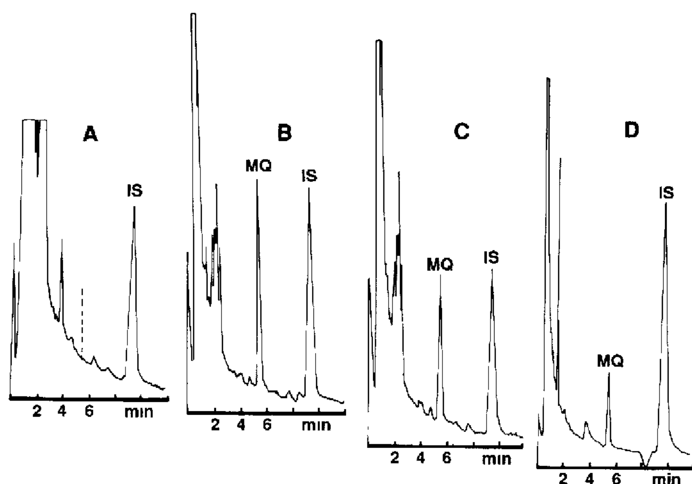

Fig. 4. Gas chromatogram of mefloquine (MQ) and internal standard (IS) after phosgene derivatization. (A) Blank whole blood; (B) whole blood adding 2.0 μmol/l MQ; (C) patient's whole blood sample containing 1.7 μmol/l MQ; (D) patient's capillary blood extracted from dried spots on filter paper containing 1.3 μmol/l MQ. GC conditions as in Experimental.

the assay of MQ in blood and plasma. Aspiration of the aqueous upper phase is convenient, because it eliminates the need to transfer the organic extract to a second tube before evaporation. MTBE was substituted for methylene chloride for blood extraction from filter paper, because the plug of filter paper at the methylene chloride–aqueous layer interface makes clean aspiration of the aqueous layer impossible. MTBE can be drawn from the top and transferred to a separate tube, but if methylene chloride is used the lower phase must be transferred.

The protein precipitation and extraction conditions have been studied previously [17]. Fig. 4 shows representative GC chromatograms from the extracts of blank whole blood, whole blood spiked with MQ and patient samples from whole blood and filter-paper-collected capillary blood. No interfering peaks were observed from endogenous components of plasma, whole blood, urine or dried blood extract.

The use of MTBE as extracting and derivatizing solvent for the capillary blood samples collected on filter paper sometimes gave a series of standard and sample chromatograms with one additional peak each corresponding to MQ and I.S. and eluting just before the corresponding expected peak. The early-eluting additional peak attributed to the second MQ-derived compound was shown to be proportional in peak height to the amount of MQ in standards. Further work is underway to characterize the products resulting from phosgene derivatization under various reaction conditions. The formation of the derivatives of MQ and I.S. giving the additional peaks may be avoided by use of methylene chloride as solvent in extraction and derivatization of filter-paper-collected blood samples.

Precision and recovery

The precision and recovery are good over the concentration range studied (Tables I and II). The recovery was nearly 90% when MQ was extracted with MTBE

TABLE I

RECOVERY FOR THE MQ ASSAY BY GC-ECD

Sample	Concentration (μmol/l)	Recovery (%)	*n*
Whole blood	0.50	85.5±5.3	5
	1.00	81.2±4.4	5
Plasma	0.10	90.7±9.9	10
	0.50	89.4±8.8	9
	1.00	80.5±9.7	4
	3.00	89.6±4.3	5
Whole blood (filter paper)	0.50	90.3±5.6	5
	0.75	91.4±4.8	4
	1.00	90.8±4.2	5
	3.00	96.8±3.4	6

TABLE II

PRECISION OF MQ ASSAY BY GC-ECD

Sample	Concentration (μmol/l)	Coefficient of variation (%)	*n*
Intra-assay			
Whole blood	0.50	2.4	5
	1.00	2.3	5
	3.00	1.5	5
Plasma	0.025	12.2	5
	0.10	3.9	6
	0.50	1.4	6
	3.00	1.5	5
Whole blood (from filter paper)	0.10	12.0	5
	0.50	2.1	6
	3.00	3.1	4
Inter-assay			
Plasma	1.00	4.4	8
Urine	4.47	2.1	5

containing phosgene in the presence of the cut-up filter paper. Earlier experiments in which the aqueous acid extract was separated from the filter paper before the derivitization step resulted in recoveries of ca. 50%. Diethylamine (0.1%) could also be used to elute MQ from blood spots, but diethylamine must be completely removed from the organic phase before the derivatization procedure, since phosgene reacts with amines. Hydrochloric acid (0.1 *M*) minimizes adsorption of the drugs on the glass surface to which MQ and I.S. are exposed during the assay.

Linearity

Calibration curves plotting the ratio of peak areas of MQ to the peak areas of I.S. versus concentration (0.125–8 μmol/l) were evaluated for MQ-spiked plasma, whole blood and whole blood applied to filter paper (Table III). Good linearity and a negligible y-intercept were routinely found.

Stability of MQ in blood spots on filter paper

The concentration of MQ in blood spots appears to be stable for at least seven to ten days at room temperature (20–23°C). However, the concentrations decrease somewhat after this time (Table IV). Kapetanovic et al. [7] have shown that MQ is stable in blood for seven days at temperatures ranging from −20°C to +37°C. We have used spiked plasma controls stored for ca. ten months at −20°C and one control stored for one month at +4°C. No degradation of MQ or its acid metabolite was observed. It seems likely that the lower values for MQ in filter paper blood spots stored at room temperature after seven to ten days

TABLE III

LINEARITY OF THE MQ ASSAY BY GC-ECD

Whole blood			Plasma			Filter paper		
Added (μmol/l)	Peak-height ratio	Calculated (μmol/l)	Added (μmol/l)	Peak-height ratio	Calculated (μmol/l)	Added (μmol/l)	Peak-height ratio	Calculated (μmol/l)
0.125			0.125	0.034	0.120	0.5	0.174	0.47
0.25	0 093	0.31	0.25	0.068	0.250	1.0	0.335	0.97
0.5	0 194	0 58	0.5	0.135	0.506	3.0	1.03	3.12
1.0	0 26	0.75	1.0	0.259	0.981	5.0	1.62	4.94
3.0	1 17	3.15	3.0	0.803	3.06			
5.0			5 0	1 301	4.77			
8.0	3.00	7.97	5 0					
Linear regression analysis								
Correlation coefficient	$r=0.999$			$r=0.999$			$r=0.999$	
Intercept	$a=-0\ 0243$			$a=0.00262$			$a=0.0205$	
Slope	$b=0\ 380$			$b=0.261$			$b=0.324$	

TABLE IV

STORAGE STABILITY OF MQ FILTER PAPER

Time (days)	Concentration (μmol/l)	
	Room temperature	+4°C
0	3.27±0.19	-
3	3.59±0.12	3.47±0.26
10	3.37±0.071	3.57±0.24
17	2.99±0.092	3.39±0.071
20	2.96±0.17	3.41±0.050

TABLE V

COMPARISON OF DIFFERENT BLOOD SAMPLING TECHNIQUES IN MQ ASSAY

Sample days after first dose	Concentration (μmol/l)		
	Venous blood	Filter-paper-spotted venous blood	Filter-paper-spotted capillary blood
7 (1:00 pm)*	0.51 ± 0.007	0.60 ± 0.19	0.65 ± 0.21
7 (4:00 pm)**	1.11 ± 0.035	1.04 ± 0.007	1.14 ± 0.078
8 (1:00 pm)	0.93 ± 0.021	0.87 ± 0.071	0.96 ± 0.035
10 (1:00 pm)	0.84 ± 0.042	-	0.82 ± 0.021

*Before weekly dose.
**3 h after weekly dose.

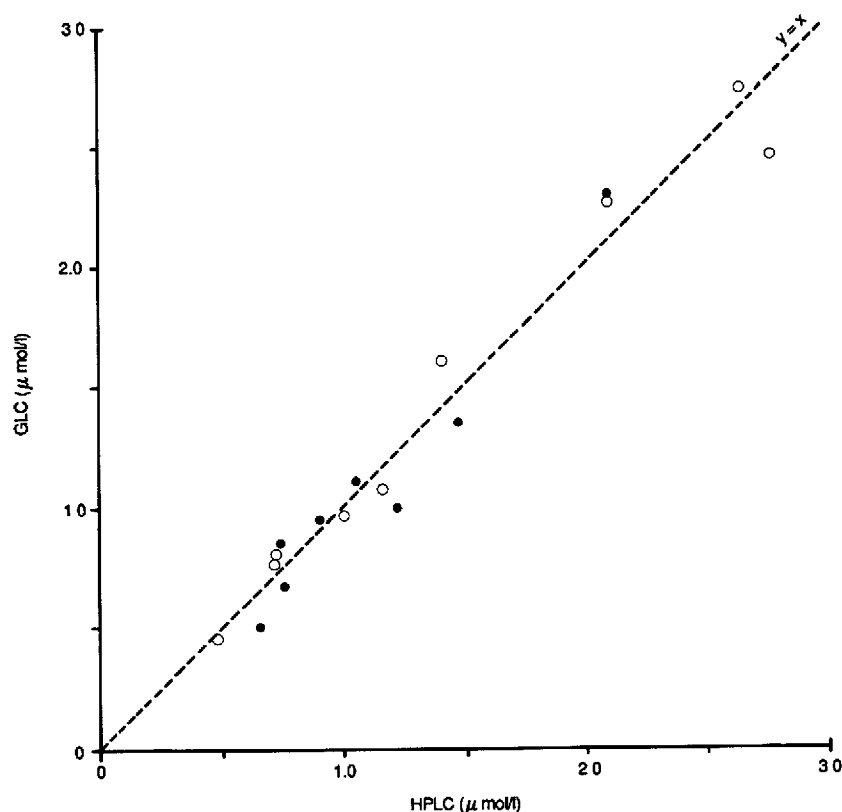

Fig. 5. Results for MQ in whole blood (●) and plasma (○) obtained by HPLC (x) and GC-ECD (y) The equation obtained was $y = 1.015x - 0.032$, and the correlation coefficient was 0.977.

compared with those stored at +4 °C could be due to irreversible occlusion of MQ in aged dried blood on filter papers with the extraction method used.

Whole blood and capillary blood comparability

There was close agreement between the results obtained from blood samples

collected by venipuncture, corresponding capillary blood samples by finger stick and collected on filter paper and whole blood applied to filter paper (Table V).

Selectivity of the present method

The present (GC) method and the HPLC method [10] for the assay of MQ were compared both in plasma and whole blood samples (Fig. 5). Seventeen samples from patients taking MQ were assayed. The results indicate that the two methods are equivalent (*t*-test) in the 0.5–3.0 μmol/l concentration range.

CONCLUSION

The method described here incorporate a simple and robust derivatization technique in which both analyte and I.S. are derivatized at the same functionality to form very stable cyclic derivatives with excellent GC properties. In previous GC methods [2–5] the silyl and perfluoroacyl derivatives formed are less stable [4,5], which can adversely affect the convenience and/or precision of the method. The sensitivity of the present method permits its application to the assay of MQ in 100-μl finger stick blood samples preserved on filter papers and transported by mail from malaria field study sites to the analytical laboratory.

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