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Determination of mefloquine in blood, filter paper-absorbed blood and urine by 9-fluorenylmethyl chloroformate derivatization followed by liquid chromatography with fluorescence detection

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ABSTRACT

We describe a method for determination of mefloquine (MQ) in 100-μl samples of urine, whole blood, and capillary blood collected on filter paper; quantification is by liquid chromatography with fluorescence detection at 475 nm of the 9-fluorenylmethyleneoxycarbonyl derivative. Whole blood and urine samples were prepared by extraction of MQ and internal standard from aqueous base with methyl *tert.*-butyl ether (MTBE), separation and evaporation of the MTBE layer, and derivatization using a solution of 9-fluorenylmethyl chloroformate in acetonitrile. Filter paper spots were immersed for 16 h in 0.1 *M* hydrochloric acid, followed by extraction with MTBE from aqueous sodium carbonate. The separated and evaporated organic layer was treated with the derivatizing solution. An aliquot was injected onto a high-performance liquid chromatography system using a C_{18} reversed-phase column and acetonitrile–water (72:28) mobile phase for filter paper spot extracts as for whole blood and urine extracts. The method has a limit of determination in blood, blood spots, and urine of 50 ng/ml with 100 μl sample size (coefficient of variation = 16%). Linearity and precision (within-day and between-day) for the method are good. The MQ derivative was isolated and characterized spectroscopically. Values for MQ concentrations in filter paper blood spots compared favorably with values found in corresponding whole blood samples analyzed by a published method.

INTRODUCTION

Mefloquine (MQ), D,L-*erythro*-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol (Fig. 1), has been extensively evaluated in recent years for prophylaxis and treatment of malaria caused by multiple-drug-resistant strains of *Plasmodium falciparum* [1]. This drug has been widely used to treat drug-resistant cases of *P. falciparum* malaria in southeast Asia and is licensed for sale in several

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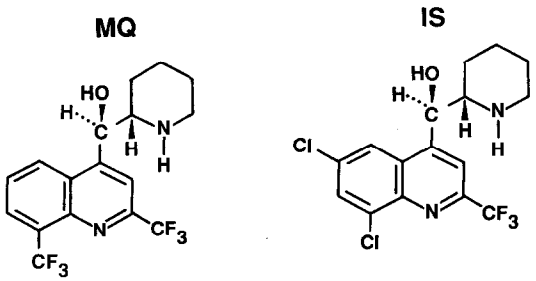

Fig. 1. Structures of mefloquine (MQ) and the internal standard (IS).

European countries, including Switzerland, France, The Netherlands, and Germany. MQ was licensed for sale in the United States in 1989. The increasing incidence and spread of strains of *P. falciparum* resistant to older antimalarial drugs has sparked an increasing need for MQ. Continuing field studies of the efficacy and safety of MQ regimens require efficient means of sample collection and transport, and sensitive, selective methods for determination of blood concentrations of this drug.

High-performance liquid chromatographic (HPLC) and gas chromatographic (GC) methods of assay of MQ in body fluids have been recently reviewed [2]. GC methods using derivatization of the polar alcohol and secondary amine groups have permitted sensitive quantification using electron-capture detection (ECD) [3–7]. One of these methods quantifies MQ in 100-μl quantities of blood absorbed and preserved on filter paper [7]. A supercritical fluid chromatography (SFC) method with ECD has been recently described for quantification of MQ with no derivatization and with sufficient sensitivity to assay MQ in 100-μl quantities of finger-stick capillary blood [8].

Methods for quantifying MQ using HPLC with ultraviolet (UV) spectrophotometric detection are less sensitive than methods with ECD and typically require approximately 500 μl of blood or plasma [9–12]. Here we describe a method using derivatization with 9-fluorenylmethyl chloroformate to yield a 9-fluorenylmethyleneoxycarbonyl (FMOC) derivative of MQ that permits analysis of 100-μl quantities of finger-stick capillary blood preserved on filter paper by HPLC with fluorescence detection.

EXPERIMENTAL[a]

Chemicals

Sodium phosphate, tribasic dodecahydrate (98%), 9-fluorenylmethyl chloroformate (97%), *n*-butyl chloroformate, benzyl chloroformate, and *n*-butylamine

[a] Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

were supplied by Aldrich (Milwaukee, WI, U.S.A.). Methyl *tert.*-butyl ether (MTBE), acetonitrile, *n*-hexane, ethyl acetate, and methylene chloride, all HPLC grade, were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Methanesulfonic acid was from Eastman Organic Chemicals (Rochester, NY, U.S.A.). MQ · HCl standard and the internal standard (I.S.), D,L-*erythro*-α-(2-piperidyl)-2-trifluoromethyl-6,8-dichloro-4-quinolinemethanol, were gifts from Walter Reed Army Institute of Research (Washington, DC, U.S.A.). All other chemicals were reagent grade or better.

Chromatography

The liquid chromatographic (LC) system consisted of a Spectra-Physics SP8700 tertiary gradient pump (San Jose, CA, U.S.A.), a Rheodyne 7125 injector with 20-μl sample loop (Cotati, CA, U.S.A.), a Bio-Rad LC column heater (Richmond, CA, U.S.A.), a Beckman 150 mm × 2.0 mm I.D. C_{18} reversed-phase column (5 μm particle diameter) (Irvine, CA, U.S.A.), a Hewlett-Packard 1046A fluorescence detector (Palo Alto, CA, U.S.A.), and a Hewlett-Packard 3392A integrator–printer–plotter. The mobile phase was acetonitrile–water (72:28). Flow-rate was 0.8 ml/min, and column temperature was 50°C. Excitation and emission wavelengths for fluorescence detection were 260 and 475 nm, respectively.

Analysis of whole blood, filter paper-absorbed blood spot, and urine samples

Whole blood. I.S. (80 μl of 5.6 ng/μl in 0.001 *M* HCl) was added to 15-ml screw-cap centrifuge tubes containing 100-μl volumes of sample or fortified blood standards. Volumes of an aqueous solution of MQ salt (0.503 μg/ml as base) were added to drug-free blood to give concentrations of 0, 50.3, 251.5, 503, 754.5, and 1006 ng/ml MQ in blood. MTBE (3 ml) and 20% aqueous $Na_3PO_4 \cdot 12H_2O$ (0.5 ml) were added to each tube. Tubes were capped, vortex-mixed for 30 s and centrifuged (600 *g*) to separate layers. The MTBE layer was transferred by Pasteur pipet to a clean centrifuge tube and evaporated to dryness using a 55°C water bath and a gentle stream of nitrogen. A 50-μl volume of 9-fluorenylmethyl chloroformate (3 μg/μl in acetonitrile) was added to each residue. The tubes were heated for 5–10 min in a 55°C water bath; 15-μl volumes of the acetonitrile solutions were injected onto the LC system for quantitation.

Filter paper-absorbed blood spots. Standard blood spots (MQ-fortified, originally drug-free blood) and sample blood spots were cut into small pieces and added to 15-ml glass screw-cap centrifuge tubes. A 100-μl volume of I.S. (5.6 ng/μl) was added to each tube followed by 2 ml of 0.1 *M* HCl. Each tube was vortex-mixed for 15 s and allowed to stand overnight. Sodium carbonate (1.2 ml of 0.4 *M* solution) and MTBE (4 ml) were added, and capped tubes were rotated for 15 min and centrifuged at 600 *g*. MTBE layers were transferred to labeled, clean 15-ml centrifuge tubes and the samples and standards evaporated to dryness. Derivatization and HPLC quantification continued from this point as for whole blood samples.

Urine. The procedure was similar to that for whole blood. However, 200 μl of 5.6 ng/μl aqueous I.S. solution were added to samples and standards; standards ranged from 0 to 3.0 μg/ml MQ, and 100 μl of the derivatization solution were used.

Volunteer study

A 74-kg male volunteer ingested a single dose of MQ · HCl (13.5 mg/kg as base). Blood and urine samples were collected at intervals, including paired samples at fourteen days.

Comparison of filter paper-absorbed blood spot results for MQ with those from a reference HPLC–UV assay

Blood samples were from a study in Tanzania [12] in which a group of children with uncomplicated malaria received 250 mg of MQ. The samples were drawn on days 1 and 7 after the first dose. For each blood sample taken, 100-μl volumes of capillary blood were absorbed on filter paper and dried, while separate 100-μl volumes of capillary blood were diluted 1:1 with water in 2-ml polypropylene tubes. Paired samples were analyzed by two different methods. The samples on filter paper were analyzed as described above for filter paper blood spots. The 100-μl volumes of blood, added to 100 μl of water in polypropylene tubes, were stored at −20°C and then analyzed by a previously described method [13]. The results of analysis of the sets of paired samples were compared.

Isolation of MQ derivative for NMR and fluorescence spectrometric analysis

A ten-fold molar excess of 9-fluorenylmethyl chloroformate was added to a solution of 50 mg of MQ in 2 ml of acetonitrile in a 15-ml screw-cap centrifuge tube and heated for 10 min in a water bath. The acetonitrile was evaporated under a gentle flow of nitrogen and the residue dissolved in 1 ml of MTBE. Three 100-μl aliquots were sequentially streaked onto a 10 cm × 2.5 cm Empore flexible silica thin-layer chromatographic (TLC) sheet (Analytichem International, Harbor City, CA, U.S.A.) with drying of solvent after addition of each aliquot. Two other sheets were streaked in the same way. All were eluted with 20% ethyl acetate in hexane. Streaks of the product (R_F = 0.5) were visualized as a blue fluorescence under 254 nm (or 365 nm) UV radiation (Spectroline Model ENF-240C, Spectronics, Westbury, NY, U.S.A.) The streaks were cut from the sheets with scissors and extracted with methylene chloride, which was then evaporated. The partially purified sample was dissolved in 0.2 ml of acetonitrile. The analytical LC system was used to separate and isolate the derivative with repetitive injections containing about 1 mg of partially purified product each. Collections were combined, and the resulting solution was placed in a 55°C water bath under a gentle stream of nitrogen. The solution clouded as the acetonitrile evaporated, leaving the aqueous portion of mobile phase, in which the derivative is insoluble. The cloudy aqueous residue was extracted with ethyl acetate. The or-

ganic phase was evaporated, leaving a residue of 7 mg of product. An additional iteration of the LC purification yielded 5 mg of product of high purity. Two 2-ml C^2HCl_3 (99.8 mol-%, Aldrich) addition/evaporation cycles were used to remove residues of ethyl acetate so that the sample could be characterized by proton nuclear magnetic resonance (NMR), UV absorption, and fluorescence spectroscopy.

NMR spectroscopy

The 300-MHz proton spectra were obtained with a Varian XL-300 spectrometer, equipped with a 7.0-tesla superconducting magnet and XL Series software, version 6.1 (Varian Assoc., Palo Alto, CA, U.S.A.). The spectra were obtained in [^{2}H]chloroform and measured in parts per million (ppm) relative to tetramethylsilane with residual [^{1}H]chloroform signal as reference.

UV absorption and fluorescence spectroscopy

UV absorption spectra of the isolated FMOC-MQ derivative diluted in methanol were obtained at room temperature using a Beckman Model DU-7 UV–VIS spectrophotometer (Irvine, CA, U.S.A.). Fluorescence spectra were also obtained in methanol. The apparatus used for fluorescence spectroscopy was constituted as follows: the excitation system for fluorescence spectroscopy consisted of a Hanovia 1000W Xenon arc lamp, a quartz focusing lens, a 10-cm water filter, a Bausch and Lomb high-intensity grating monochromator, and a second quartz lens. The sample was mounted in a dark box. Emitted light was detected at 90°C to the excitation beam after passing through a quartz lens and a SPEX 1680 double monochromator. This emitted light was detected by an RCA 1P-28 photo-multiplier tube and amplified. Further signal-processing was achieved with an IBM PS/2 Model 30 computer with an Omega DAS-16F interface board and Lab Tech Notebook (Wilmington, MA, U.S.A.) software. Excitation monochromator slits were set at 3.0 mm entrance and 2.5 mm exit, yielding a bandpass of about 8 nm (full width, half maximum). Emission monochromator slits were set at 2.0 mm, entrance and exit. Experiments were carried out with excitation wavelengths of 260 and 295 nm.

Comparison of fluorescence intensities of MQ and three derivatives

A 0.6-ml volume of aqueous MQ · HCl (0.52 mg/ml as base) was added to a 15-ml screw-cap centrifuge tube. MTBE (6 ml) and 20% sodium phosphate tribasic dodecahydrate were added, followed by vortex-mixing (1 min) and centrifugation (600 *g*). Aliquots (1 ml) of the MTBE layer were transferred to each of four separate tubes. The tubes were placed in a 60°C water bath and the solvent evaporated with a gentle stream of nitrogen. To three of the tubes were added 0.2 ml of 3 mg/ml (in acetonitrile) of either 9-fluorenylmethyl chloroformate, *n*-butyl chloroformate, or *n*-benzyl chloroformate. These tubes were capped, vortex-mixed for 20 s, and placed in a 60°C water bath for 10 min. The MQ residue in the

fourth tube was dissolved in 0.2 ml of acetonitrile. The three solutions with MQ derivatives were analyzed by HPLC using a mobile phase comprised of 33% of 0.1 *M* aqueous methanesulfonic acid (adjusted to a pH of 2.5 with *n*-butylamine) in acetonitrile. MQ was analyzed with a mobile phase of 60% of 0.1 *M* aqueous methanesulfonic acid (adjusted to pH 2.5 with *n*-butylamine) in acetonitrile. Maxima for excitation and emission wavelengths for the four compounds were determined by iterative injections at systematically varied excitation and emission wavelengths. The same experiments were conducted for the three derivatives in a mobile phase of water–acetonitrile (33:67).

RESULTS AND DISCUSSION

Characterization of the method

Chromatograms for MQ assay in blood (Fig. 2) and urine (Fig. 3) show the short analysis time possible with the method, partially because of its selectivity. Chromatograms from assay of MQ in filter paper-absorbed, finger-stick blood spots were essentially the same as for blood samples not preserved on filter paper (Fig. 2).

Standard curve data for assay of MQ in blood showed good linearity using peak-height ratios for calculation of results (Table I). While use of peak-area ratios showed comparable linearity, the within-day coefficient of variation (C.V.) at the limit of determination was appreciably better for peak-height ratio calculations (14.4% *versus* 28.5%). Within-day precision at 503 ng/ml was 2.90% (n =

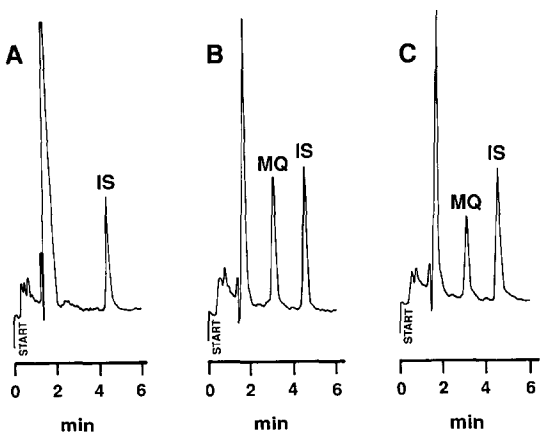

Fig. 2. Chromatograms from application of the method to (A) an extract of 0.1-ml drug-free blood containing 80 μl of 5.6 ng/μl internal standard (IS) solution alone, (B) an extract of a 0.1-ml drug-free blood standard containing 503 ng/ml mefloquine (MQ), and (C) an extract of a 0.1-ml blood sample from a volunteer fourteen days after a 13.5 mg/kg dose (as base) of MQ · HCl; the concentration of MQ in blood was 342 ng/ml. An extract of a predose blood sample showed no peaks at the retention times corresponding with elution of MQ or IS.

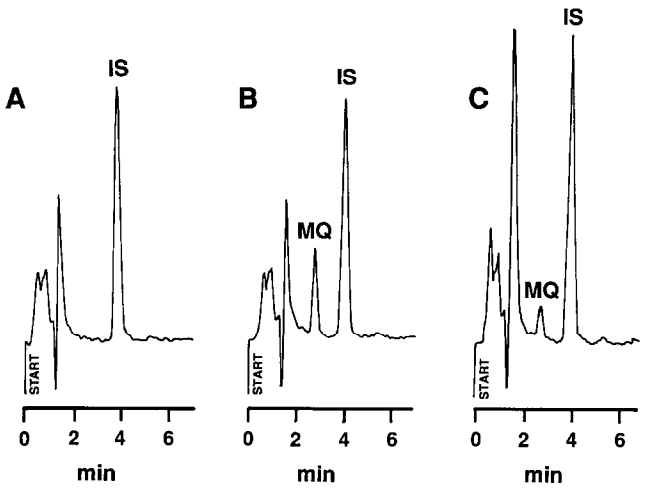

Fig. 3. Chromatograms from application of the method to (A) an extract of a 0.1-ml drug-free urine containing 200 μl of 5.6 ng/ml internal standard solution alone, (B) an extract of a 0.1-ml drug-free urine standard containing 1000 ng/ml MQ, and (C) an extract of a 0.1-ml urine sample from a volunteer fourteen days after a 13.5 mg/kg dose (as base) of MQ · HCl; the concentration of MQ in urine was 250 ng/ml. An extract of a predose urine showed no peaks at the retention times corresponding with elution of MQ or IS. The urine samples were analyzed at a later time than the blood samples; the slight variation in retention times is ascribed to a change in column activity.

4) for peak-height quantitation *versus* 3.98% (n = 4) for peak-area values. The limit of determination was 50 ng/ml. The detection limit, defined as the concentration of MQ whose extract yields a peak three times peak-to-peak baseline noise, was also 50 ng/ml.

TABLE I

STANDARD CURVE AND PRECISION DATA BY PEAK-HEIGHT RATIO FOR MQ IN 100-μl WHOLE BLOOD STANDARDS

Concentration added (ng/ml)	Peak-height ratio (y)	Concentration calculated (x') (ng/ml)
0	0	−18.7
50.3	0.131	60.2
251.5	0.457	256
503.0	0.880	511
754.5	1.294	760
1006	1.685	996

$y = mx + b$; $m = 1.66 \cdot 10^{-3}$; $b = 3.1 \cdot 10^{-2}$; $r = 0.9996$; $n = 6$

C.V. (within-day, 50.3 ng/ml) = 14.4% (n = 4); C.V. (within-day, 503 ng/ml) = 2.90% (n = 4); C.V. (injection repeatability, 503 ng/ml) = 3.99% (n = 4)

TABLE II

STANDARD CURVE AND PRECISION DATA BY PEAK-HEIGHT RATIO FOR MQ IN 100 μl OF BLOOD PRESERVED ON FILTER PAPER

Concentration added (ng/ml)	Peak-height ratio (y)	Concentration calculated (x') (ng/ml)
0	0	−10.5
47	0.066	46.0
94	0.125	96.5
189	0.25	203
377	0.46	383
755	0.87	734
1136	1.35	1145

$y = mx + b$; $m = 1.168 \cdot 10^{-3}$; $b = 0.01232$; $r = 0.9960$; $n = 7$

C.V. (within-day, 57.5 ng/ml) = 15.7% ($n = 6$); C.V. (within-day, 764 ng/ml) = 2.46% ($n = 5$); C.V. (between-day, 425 ng/ml) = 11.1% ($n = 6$)

The standard curve for finger-stick blood preserved on filter paper showed good linearity and precision using peak-height ratios (Table II). The limit of determination was 50 ng/ml, and the between-day C.V. was 11.1% at the 425 ng/ml concentration. A representative urine standard curve yielded the linear least-squares line $y = 0.7660\ x + 0.0490$, $n = 5$, $r = 0.9956$.

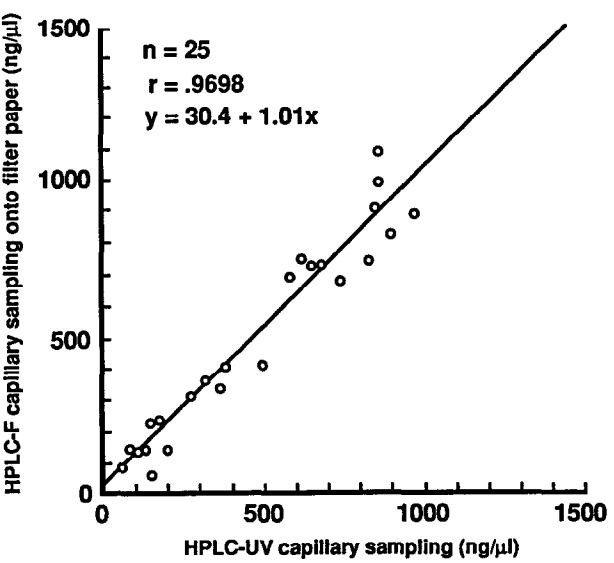

Fig. 4. Results of assay of MQ by HPLC–fluorescence in 0.1-ml volumes of blood preserved on filter paper compared with those obtained from 0.1-ml volumes of whole blood by HPLC–UV.

MQ in blood spots by HPLC–fluorescence versus MQ in whole blood by HPLC–UV

Fig. 4 shows good agreement between MQ values in paired samples run by the HPLC–fluorescence method for filter paper blood spots and a reference HPLC–UV method applied to 100-μl whole blood samples. The sensitivity of the HPLC–fluorescence method permits the convenience of finger-stick blood sampling in the field with filter paper sample preservation for transport to the laboratory. Techniques for finger-stick blood sampling and preservation on filter paper have been described in an earlier report [7]. Studies have shown that concentration values for MQ on filter paper blood samples fall 30% with seven-week storage at 25°C but less than 5% over the same time period at 4°C (ref. 7 and unpublished results, C. R. Allen and Y. Bergqvist). Therefore, filter paper blood spots for MQ assay should be kept in a refrigerator or freezer whenever possible between sampling and analysis. Further studies are indicated to characterize the possible means of inhibiting slow MQ loss during room temperature storage of filter paper blood samples.

Spectroscopic characterization of the FMOC-MQ derivative

The ^{1}H NMR spectrum of the 5-mg quantity of purified derivative was consistent with the structure of the expected N-FMOC derivative (Fig. 5). Integration

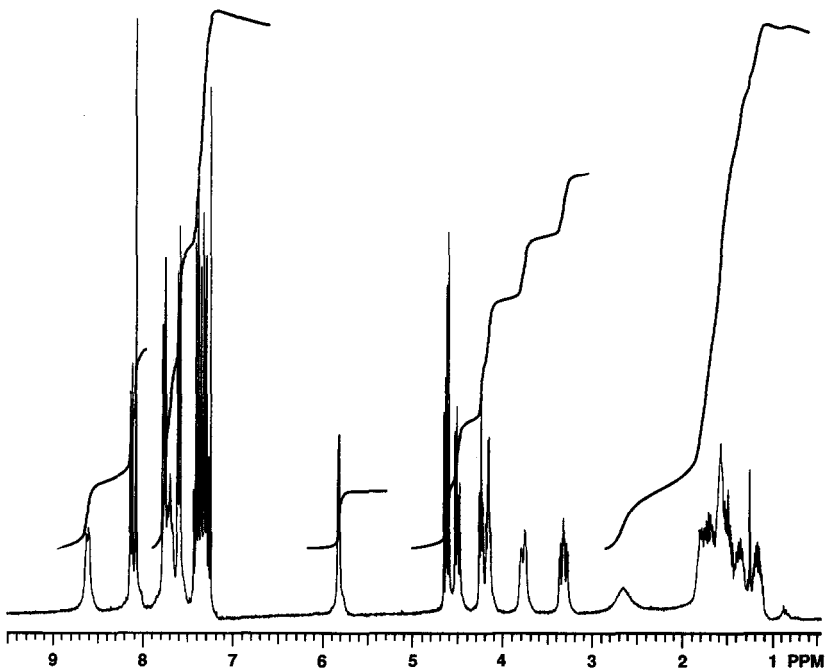

Fig. 5. ^{1}H NMR spectrum of fluorescent FMOC-MQ derivative.

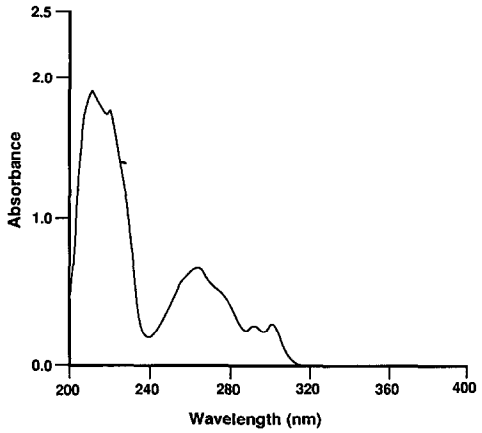

Fig. 6. Qualitative UV absorption spectrum in methanol of fluorescent FMOC-MQ derivative at room temperature.

of peaks confirmed monosubstitution. Of the 26 protons in the molecule, 12 are aromatic and are found above 7 ppm. Proton resonances due to the quinoline moiety of MQ are a multiplet at around 7.7 ppm (6 position), a singlet at 8.10 ppm (3 position), a doublet at 8.15 ppm (7 position) and a broad doublet at 8.65 ppm (5 position). The other aromatic resonances seen are attributable to the 8 protons of the fluorenyl moiety. Of the remaining 14 protons in the molecule, 3

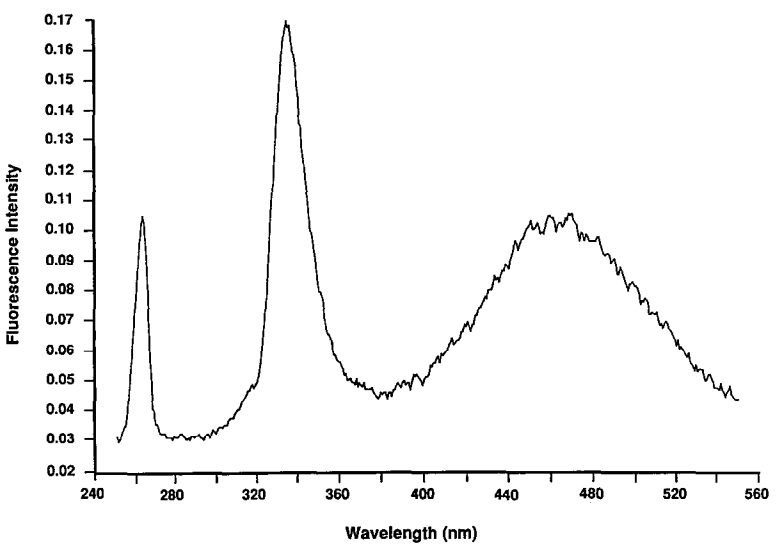

Fig. 7. Qualitative fluorescence spectrum in methanol of fluorescent FMOC-MQ derivative at room temperature with an excitation wavelength of 260 nm. The peak at 260 nm is caused by stray excitation light.

TABLE III

RELATIVE FLUORESCENCE INTENSITIES OF MQ AND THREE DERIVATIVES

Compound	Excitation maximum (nm)	Emission maximum (nm)	Area counts per μg	Relative fluorescence intensity
MQ[a]	260	410	128 690	1.00
BzOC-MQ[b]	280	455	216 415	1.68
BuOC-MQ[b]	280	455	218 535	1.67
FMOC-MQ[b]	250	485	3 793 950	29.48

[a] Mobile phase: 0.1 *M* aqueous methanesulfonic acid (adjusted to pH 2.5 with butylamine)–acetonitrile (60:40).

[b] Mobile phase: 0.1 *M* aqueous methanesulfonic acid (adjusted to pH 2.5 with butylamine)–acetonitrile (33:67).

are due to the fluorenyl moiety and 11 to the mefloquine moiety. Complete spectral assignments for all resonances cannot be made with certainty without additional experiments.

The UV absorption spectrum of the derivative is shown in Fig. 6. Excitation at 260 nm yields a fluorescence spectrum with two bands, one centered at 330 nm and another at 475 nm (Fig. 7). The band at the lower wavelength is characteristic of FMOC derivatives [14], whereas the 475-nm band is ascribed to the quinoline moiety of the MQ portion of the molecule. MQ itself has a weak fluorescence under reversed-phase HPLC conditions that we have not found to be analytically useful. However, not only is the 475-nm fluorescence of FMOC-MQ sufficiently intense for sensitive quantitation of MQ, its use also avoids interference from derivatizing reagents and by-products, thus simplifying sample preparation and reducing chromatographic requirements.

Table III shows that derivatization of MQ with *n*-butyl chloroformate or benzyl chloroformate to introduce the corresponding non-fluorescent groups yields products with fluorescence intensities similar to those of MQ itself and about 30-fold less than those of FMOC-MQ. Indeed, the approximately 30-fold increase measured for FMOC-MQ relative to MQ itself is an underestimation, since the derivatization reaction is less than 100% efficient. The FMOC moiety in the MQ derivative appears to interact intramolecularly with the substituted quinoline structure to produce the strong fluorescence at 475 nm. Such phenomena have been demonstrated in other systems in which two fluorophores in a molecule interact through space, even though they are electronically insulated from each other along intervening saturated bonded chains [15]. The chromatographic system containing methanesulfonic acid and butylamine in the mobile phase permitted comparison of the fluorimetric properties of MQ and is derivatives under similar conditions.

MQ itself did not elute in the water–acetonitrile mobile phase of the method

TABLE IV

RELATIVE FLUORESCENCE INTENSITIES OF THREE MQ DERIVATIVES

Mobile phase: water–acetonitrile (33:67).

Compound	Excitation maximum (nm)	Emission maximum (nm)	Area counts per μg	Relative fluorescence intensity
BzOC-MQ	280	455	214 190	1.00
BuOC-MQ	280	455	234 150	1.09
FMOC-MQ	250	475	9 069 700	42.3

described here. Comparison of the relative fluorescence intensities resulting from chromatography of the three MQ derivatives show that the value for FMOC-MQ is 42 times that for benzyloxycarbonyl MQ (BzOC-MQ) (Table IV), assuming equivalent conversions. Comparison of the area counts per microgram for MQ in Table III and FMOC-MQ in Table IV indicates a lower limit of about a 70-fold fluorescence enhancement. The basis for cross-comparison of data between Tables III and IV is the similarity of fluorescence intensities for BzOC-MQ in the two tables. Delineation of details of the enhancement mechanism in the present case will require further studies.

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REFERENCES

1 *Advances in Malaria Chemotherapy*, WHO Technical Report Series, No. 711, World Health Organization, Geneva, 1984.
2 Y. Bergqvist and F. C. Churchill, *J. Chromatogr.*, 434 (1988) 1–20.
3 T. Nakagawa, T. Higuchi, J. L. Haslam, R. D. Shaffer and D. W. Mendenhall, *J. Pharm. Sci.*, 68 (1979) 718–721.
4 D. E. Schwartz and U. B. Ranalder, *Biomed. Mass Spectrom.*, 8 (1981) 589–592.
5 P. Heizmann and R. Geschke, *J. Chromatogr.*, 311 (1984) 411–417.
6 D. Dadgar, J. Climax, R. Lambre and A. Darragh, *J. Chromatogr.*, 337 (1985) 47–54.
7 Y. Bergqvist, F. C. Churchill and D. L. Mount, *J. Chromatogr.*, 428 (1988) 281–290.
8 D. L. Mount, L. C. Patchen and F. C. Churchill, *J. Chromatogr.*, 527 (1990) 51–58.
9 J. M. Grindel, P. F. Tilton and R. D. Shaffer, *J. Pharm Sci.*, 66 (1977) 834–836.

10 I. M. Kapetanovic, J. D. Digiovanni, J. Bartosevich, V. Melendez, J. von Bredow and M. Heiffer, *J. Chromatogr.*, 277 (1983) 209–215.
11 P. J. Arnold and O. V. Stetten, *J. Chromatogr.*, 353 (1986) 193–200.
12 W. Hellgren, *Dissertation,* Karolinska Institute, Stockholm, 1990.
13 Y. Bergqvist, V. Hellgren and F. C. Churchill, *J. Chromatogr.*, 432 (1988) 253–263.
14 T. Y. Chou, C. X. Gao, S. T. Colgan, I. S. Krull, C. Dorschel and B. Bidlingmeyer, *J. Chromatogr.*, 454 (1988) 169–183.
15 E. L. Wehry, In G. G. Guilbault (Editor), *Practical Fluorescence,* Marcel Dekker, New York, 1973, p. 86.