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Determination of mefloquine in blood by supercritical fluid chromatography with electron-capture detection

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SUMMARY

Supercritical fluid chromatography (SFC) with electron-capture detection is described for the sensitive quantification of mefloquine in 0.1-ml blood samples. The method is internally standardized and incorporates partitioning into methyl *tert.*-butyl ether (MTBE) from aqueous base, back-extraction into dilute aqueous acid and final partitioning into MTBE from aqueous base. SFC conditions include a silica-gel-packed, glass-lined steel column and a mobile phase of 0.15% *n*-butylamine and 1% methanol in supercritical *n*-pentane. The method has a detection limit of 7.5 ng/ml in 0.1-ml blood samples and exhibits good linearity and precision. The method compares favorably with a published high-performance liquid chromatographic procedure in the analysis of blood from volunteers who received mefloquine hydrochloride (15 mg as base per kg body weight).

INTRODUCTION

Mefloquine (MQ), D,L-*erythro*-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol (Fig. 1), is increasingly used to treat malaria caused by multiple-drug-resistant strains of *Plasmodium falciparum* [1]. High-performance liquid chromatographic (HPLC) and gas chromatographic (GC) methods have been developed to assay MQ in whole blood and plasma in support of studies of this important antimalarial. These methods have been discussed in a recent review [2].

The most sensitive approach to the quantification of MQ in body fluids has

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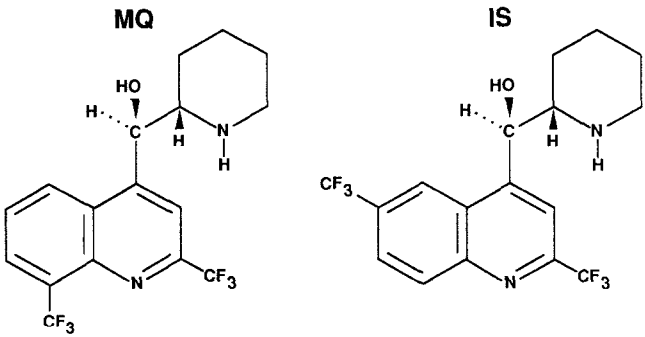

Fig. 1. Structures of mefloquine (MQ) and the internal standard (IS).

been GC with electron-capture detection (ECD) [3–6]. These methods have used derivatization by silylation and/or perfluoroacylation [3–5] and, more recently, by cyclization with phosgene [6]. Replacement of the active hydrogens with non-polar groups results in derivatives with properties much better for GC than those of MQ, and the electron affinity remains intact. However, derivatization adds steps to the analysis and adds derivatizing agents and reaction products to the process; if not effectively removed, these substances can adversely affect the efficiency of capillary columns and the sensitivity of the ECD.

Use of supercritical fluid chromatography (SFC), recognized as a separation technique since 1962 [7], is becoming routine as instruments for SFC become commercially available [8]. Supercritical fluid mobile phases have solvating power, as have mobile phases in liquid chromatography, and separation efficiencies for SFC are between those for HPLC and GC. Gas phase detectors such as a flame-ionization detector [8] or an electron-capture detector [9] can be used for SFC. Even so, limited use has been made of SFC–ECD to date [9]. We describe a sensitive SFC–ECD method in which MQ is assayed without derivatization in extracts of 0.1-ml blood samples.

EXPERIMENTAL[a]

Standards

MQ·2HCl was a gift from the Walter Reed Army Institute of Research (Washington, DC, U.S.A.) as was the internal standard D,L-*erythro*-α-(2-piperidyl)-2,6-bis(trifluoromethyl)-4-quinolinemethanol dihydrochloride (Fig. 1). The stock solution contained 503 μg/ml MQ in 0.001 *M* hydrochloric acid. This solution was diluted 1 : 1000 to give a working solution of 503 ng/ml (0.503 ng/μl) MQ in 100 ml of 0.001 *M* hydrochloric acid.

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

Reagents and solvents

Nanograde-quality *n*-pentane was from Mallinckrodt (Paris, KY, U.S.A.). Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) provided methanol, methyl *tert.*-butyl ether (MTBE) and isooctane, all HPLC grade. Trisodium phosphate dodecahydrate and *n*-butylamine, 99%, were from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals used were of reagent grade or better.

Blood samples

A subset of blood samples collected during a volunteer pharmacokinetic study [10] of an MQ regimen was analyzed by SFC–ECD and by a published HPLC method with ultraviolet (UV) spectrophotometric detection [11]. Each volunteer had been given a single dose of MQ·2HCl (15 mg as base per kg of body weight).

Apparatus

We constructed the SFC–ECD system from a Model μLC-500 high-pressure syringe pump (Isco, Lincoln, NE, U.S.A.), a Model 7125 HPLC injector with a 10-μl loop (Rheodyne, Cotati, CA, U.S.A.), a Model 3700 gas chromatograph with a ^{63}Ni electron-capture detector (Varian Assoc., Walnut Creek, CA, U.S.A.) and a 200 mm × 0.75 mm I.D. glass-lined steel column (Scientific Glass Engineering, Austin, TX, U.S.A.) packed with 7-μm Zorbax-BP spherical silica (Du Pont, Wilmington, DE, U.S.A.). We packed the column according to the procedure of Meyer and Hartwick [12].

The HPLC injector was mounted above the GC injector port and connected to the column (which was inside the GC oven) with 0.178 mm I.D. stainless-steel tubing through the GC injection port orifice. The exit end of the silica column was joined to the detector with 30 cm × 40 μm I.D. (0.375 mm O.D.) fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, U.S.A.). The column and tubing were connected with a 1.6–0.4 mm I.D. vespel/graphite reducing ferrule (Alltech Assoc., Deerfield, IL, U.S.A.). A 1.6–0.5 mm I.D. graphite reducing ferrule (Scientific Glass Engineering) was used to connect the tubing to a 6.35 mm capillary tee conversion adapter (Scientific Glass Engineering) which in turn was interfaced with the detector. Nitrogen make-up gas was supplied at a flow-rate of 30 ml/min through the arm of the tee adapter. PTFE tubing forced over the exit nozzle of the detector carried the effluent to a fume hood.

Analysis of blood samples

Standards were prepared in 15-ml screw-capped glass centrifuge tubes by adding 75 μl of 0.6 ng/μl internal standard solution, 100 μl of blank blood and appropriate quantities of 0.503 ng/μl as base of MQ·2HCl in 0.001 *M* hydrochloric acid to achieve concentrations of 0, 50.3, 251.5, 503 and 1006 ng/ml MQ in blood. Sample bloods were twice drawn into and expelled from the tips of

the Pipetman P-200 repeating pipette (Rainin, Woburn, MA, U.S.A.), then added to centrifuge tubes that contained 75 μl of internal standard solution. We added 0.5 ml of aqueous 20% sodium phosphate and 3.0 ml of MTBE to samples and standards. Each tube was capped, vortex-mixed for 30 s and centrifuged at 600 *g* for 1 min. We transferred each organic layer to a clean centrifuge tube that contained 3 ml of 0.1 *M* hydrochloric acid. *n*-Hexane (3 ml) was added, and the tubes were vortex-mixed for 30 s and centrifuged (600 *g*) for 1 min. Each organic layer was aspirated by Pasteur pipette and discarded. We added MTBE (6 ml) and aqueous 20% sodium phosphate (1 ml) to each tube; the tubes were capped, vortex-mixed for 30 s and centrifuged at 600 *g* for 1 min. The organic layer was transferred to a clean centrifuge tube and evaporated to dryness with a gentle flow of dry nitrogen and a 60°C water bath. Each sample and standard was reconstituted with 100 μl of 0.15% butylamine in MTBE–isooctane (15:85) and analyzed by SFC–ECD.

Chromatographic procedure

A freshly packed column was installed and conditioned 4–6 h at 300°C with flow of mobile phase [supercritical pentane containing *n*-butylamine (0.15%) and methanol (1%) as modifiers] at a pressure of 2100 kPa. The mobile phase may be passed through the electron-capture detector during the conditioning procedure without deleterious effect on the subsequent performance of the detector. During analytical operation of the system, the column and transfer lines were held at 210°C and the detector at 350°C. At the beginning of each day the SFC system was allowed to equilibrate with flow for 10–20 min before analysis was begun. Concentrated sample and standard extracts (10 μl) were injected into the SFC system for analysis. Retention times for internal standard and MQ were 4 and 8 min, respectively.

Recovery study

A 565 μl/ml solution of MQ base in MTBE was diluted 1:10 in methanol. A 1-ml volume of this solution was diluted to 100 ml with 0.01 *M* hydrochloric acid (solution A). A second 1-ml quantity of solution was diluted to 100 ml with 0.15% *n*-butylamine in MTBE–isooctane (15:85) (solution B). Each of three 100-μl quantities of blank blood was fortified with a 100-μl quantity of solution A to give 565 ng/ml MQ in blood. These standards were carried through analysis, but quantification was by external standardization by direct comparison with solution B. Recoveries were calculated and averaged.

Reproducibility studies

Within-day reproducibility was evaluated by analysis of blood samples with MQ concentrations of about 100 and 450 ng/ml. Between-injection reproducibility was determined for concentrations of about 450 ng/ml.

RESULTS AND DISCUSSION

The back-extraction into aqueous acid was included in the sample preparation procedure to remove lipids that could have degraded column efficiency. The 3 ml of *n*-hexane added before extraction into acid greatly increased the recovery in this step. Overall recovery of MQ for the method was $68.3 \pm 5.9\%$ ($n=4$), measured at the 565 ng/ml level.

Newly prepared columns were conditioned for several hours to achieve acceptable peak shape. If peak shape was poor after the 10–20 min equilibration when use of the column was resumed after a hiatus, conditioning at 300°C for 30 min to 1 h was generally sufficient to restore efficiency.

Representative chromatograms are shown in Fig. 2. In early work, chromatograms showed a small peak near that of MQ and a larger peak at 16 min when extracts of blank blood were injected. These peaks were due to contaminants in the centrifuge tubes. Both interfering peaks disappeared after the tubes were routinely rinsed with 10 ml of 0.15% *n*-butylamine in MTBE before use. After the rinsing regimen was introduced into the method, no peaks were seen beyond the void volume after sample injection other than those due to MQ and the internal standard. The detection limit of the method was 7.5 ng/ml, which corresponds to a peak height of three times baseline noise. Peak-height ratios were used to calculate concentrations due to the adverse affect of the tailing of MQ and internal standard peaks on peak-area measurements.

Standard curve data were calculated for MQ in 100 µl of blood (Table I). Within-day and between-injection reproducibilities were measured (Table II).

Results from SFC–ECD and HPLC–UV analysis of 23 samples from the

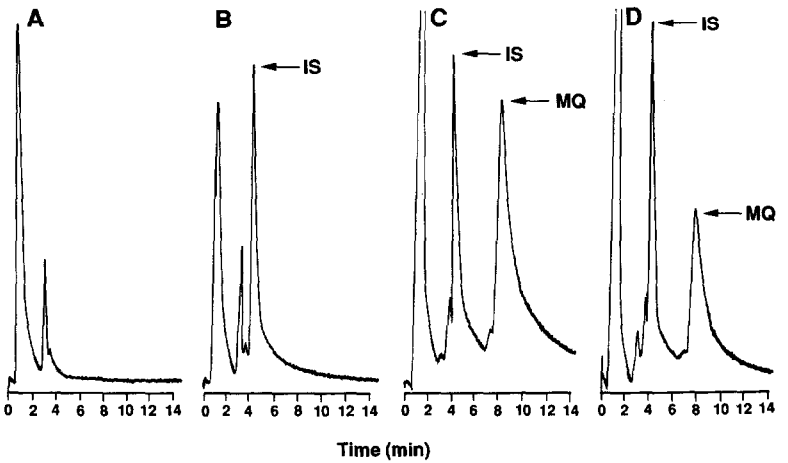

Fig. 2. SFC–ECD chromatograms for 100-µl quantities of (A) blank blood, (B) blank blood containing internal standard (IS) only, (C) blood fortified to contain 503 ng/ml MQ and (D) a blood sample from a volunteer (concentration MQ = 266 ng/ml).

TABLE I

STANDARD CURVE DATA FROM SUPERCRITICAL FLUID CHROMATOGRAPHIC ASSAY OF MEFLOQUINE IN 0.1-ml SAMPLES OF WHOLE BLOOD

Concentration added (ng/ml)	Mefloquine/internal standard peak-height ratio	Concentration found[a] (ng/ml)
0.0	0.0	0.39
50.3	0.0976	54.3
251.5	0.462	255.8
503.0	0.882	488.0
1006.0	1.830	1012.2

[a]Calculated from the least-squares line $y=mx+b$, where $m=1.81\cdot10^{-3}$ and $b=-6.99\cdot10^{-4}$; $r=0.9998$, $n=5$.

TABLE II

REPRODUCIBILITY OF SUPERCRITICAL FLUID CHROMATOGRAPHIC ASSAY OF MEFLOQUINE IN BLOOD

Sample designation	Mefloquine/internal standard peak-height ratio	Concentration found[a] (ng/ml)
Within-day reproducibility		
PS-1H	0.209	116
	0.193	107
	0.191	106
	0.178	99
	Mean ± S.D.	107 ± 7.0
	R.S.D.	6.5%
EL-14D	0.741	410
	0.757	419
	0.788	436
	0.805	446
	Mean ± S.D.	428 ± 16.2
	R.S.D.	3.8%
Between-injection reproducibility		
EL-14D-4	0.805	446
	0.815	451
	0.855	473
	0.752	416
	Mean ± S.D.	446 ± 23.5
	R.S.D.	5.3%

[a]Calculated from the least-squares line described in Table I.

volunteer study showed good agreement. The data are plotted and resulting statistical parameters shown (Fig. 3). Fig. 3 shows correlation of the methods but does not indicate which method is more accurate.

n-Pentane showed several advantages over the more commonly used carbon dioxide for the SFC analysis of MQ. It was convenient to add precise amounts of modifiers to *n*-pentane, a liquid at room temperature. Furthermore, the electron-capture detector responded less to *n*-pentane than to carbon dioxide and gave a lower chromatographic baseline and less noise with *n*-pentane than with carbon dioxide. The apparatus for SFC–ECD was relatively simple and inexpensive; it included a syringe pump, an HPLC injector and an old gas chromatograph with ECD capability. The SFC column was packed in the laboratory, and 10–20 μl of extract were injected directly into the analytical system.

SFC permits the exploitation of the electron affinity of MQ even though the drug is not directly amenable to GC because of the polar groups it contains. MQ is separated from the analogue internal standard, and each is detected in turn as it is carried by the supercritical mobile phase through the electron-capture detector. Although detector noise is greater for SFC–ECD than for GC–ECD and although the MQ peak tails appreciably, the SFC–ECD method yields a detection limit of 7.5 ng/ml in analysis of 100-μl blood samples. This limit is nearly the same as that of a published method [6] in which MQ is derivatized and quantified by GC–ECD. The sensitivity of the HPLC–UV methods for MQ is about one tenth that for the methods that use ECD, limiting the applicability of the former for analysis of finger-stick blood [2].

All three analytical approaches yield within-day and between-day precisions under 10%; the carboxylic metabolite has been quantified along with MQ by

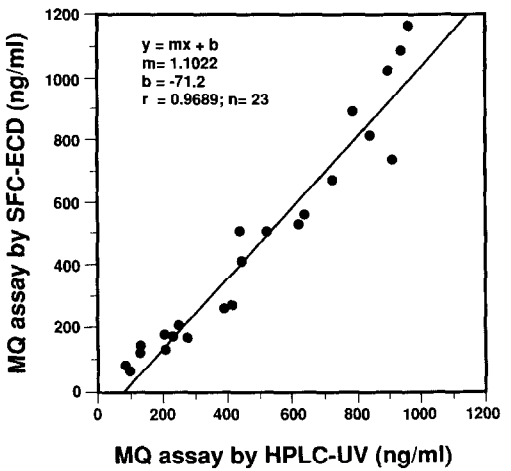

Fig. 3. Results of assay for MQ in blood by SFC–ECD compared with those obtained by HPLC–UV.

HPLC [2] but not by the methods using ECD. The SFC–ECD method described herein requires more steps in sample preparation than the other two approaches, but is more sensitive and selective than HPLC–UV and more rugged than GC–ECD, where derivatizing agents and/or their by-products can contaminate the ECD foil, requiring periodic cleaning to restore lost sensitivity. All three approaches are amenable to automating chromatographic quantification of sample extracts.

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