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GLC Determination of Whole Blood Antimalarial Concentrations

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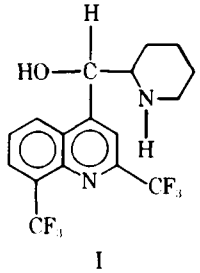
Abstract □ An assay was developed for determining mefloquine (quinolinemethanol) and pyridinemethanol derivative concentrations in whole blood. The method involved ion-pair extraction or usual solvent extraction for drug recovery from whole blood followed by trimethylsilylation. The silylated compounds were then submitted to GLC with electron-capture or flame-ionization detection. Mass spectrometry combined with GLC of the trimethylsilyl derivatives indicated that substitution of one trimethylsilyl group had occurred on the hydroxyl group. A phenyl methyl silicone column with temperature programming separated the drugs from normal blood extracts. The determination limit was 10 ng/ml of whole blood when an electron-capture detector was used with ion-pair extraction. Quantitation was achieved by using one antimalarial as an internal standard for the assay of the other. The utility of the present method was demonstrated by following the whole blood level time course after a single oral 250-mg tablet in beagle dogs.

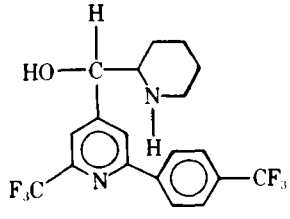
Keyphrases □ Mefloquine—analysis, GLC, blood □ Pyridinemethanol derivatives—analysis, GLC, blood □ Antimalarials—analysis, GLC, blood □ GLC, electron capture—analysis, mefloquine, pyridinemethanol derivatives, blood □ GLC, flame ionization—analysis, mefloquine, pyridinemethanol derivatives, blood

Mefloquine (I), DL-*erythro*-α-(2-piperidyl)-2,8-bis-(trifluoromethyl)-4-quinolinemethanol, is curative for drug-resistant falciparum malaria (1–3). This compound has an extremely long biological half-life and is distributed rapidly into the body tissue (4), suggesting that the amount of the drug circulating in blood is low and persistent.

DL-*threo*-α-2′-Piperidyl-2-(4-trifluoromethylphenyl)-6-trifluoromethyl-4-pyridinemethanol (II) is a new antimalarial with activity against *Plasmodium berghei* (5). Sensitive and specific assays for whole blood concentrations of both compounds were needed.



I



II

Several methods have been used for antimalarial analysis, including high-pressure liquid chromatography (HPLC) (6, 7), GLC (8), fluorometry (8), and liquid scintillation counting (9), but no reports have dealt with GLC determination of I and II in blood. Of these methods, HPLC combines better reproducibility and specificity than the fluorometry or radioactivity methods with a simple pretreatment procedure; it was used for I determination. However, HPLC sensitivity was limited by the relatively low I extinction coefficient at the readily accessible wavelengths, 280 and 254 nm; GLC combined with an electron-capture detector was expected to give more sensitive and specific measurements. This paper describes whole blood I and II determinations by electron-capture GLC.

EXPERIMENTAL

Reagents and Materials—Free bases of I and II were prepared by alkalizing the corresponding hydrochloride salts using equimolar sodium hydroxide in ethanol. The elemental analysis data (I—calc.: C, 53.97; H, 4.26; N, 7.41. Found: C, 54.29; H, 4.09; N, 7.59. II—calc.: C, 56.43; H, 4.49; N, 6.93. Found: C, 56.45; H, 4.47; N, 6.79), TLC, and GLC supported the purity of both free bases. The trimethylsilylating reagent[1] was a mixture of hexamethyldisilazane and trimethylchlorosilane dissolved in pyridine. Fresh human blood was obtained from volunteers. All other chemicals were commercially available analytical grades and were used without further purification.

GLC—The gas chromatograph[2] was equipped with an electron-capture detector and a flame-ionization detector. A U-shaped glass column (183

[1] TriSil, Pierce Chemical Co., Rockford, Ill.
[2] Varian Aerograph model 2100, Walnut Creek, Calif.

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Table I—Compound I Recovery from Spiked Blood Samples by Ion-Pair Extraction followed by Electron-Capture and Flame-Ionization GLC Determinations

Blood Level, ng/ml	Recovery (Mean ± *SD*), %[a]
1070[b]	87.7 ± 4.6
535[b]	88.3 ± 4.6
107[b]	94.0 ± 5.2
10.4[c]	92.9 ± 9.4
5.3[c]	94.4 ± 15.3
1.04[c]	100.5 ± 19.1
Average	93.0 ± 9.7

[a] From three replicate samples. [b] Flame-ionization detection. [c] Electron-capture detection.

cm × 4 mm i.d.) was packed with 3% phenyl methyl silicone gum[3] coated on a flux-calcined diatomite support[4] (100–200 mesh, acid washed, dimethylchlorosilane treated). The electron-capture detection used an 8 mCi ^{63}Ni-radiation source, a 90-v dc cell voltage, and $1.0–1.2 \times 10^{-9}$ amp standing current. For flame-ionization detection, 30 ml of hydrogen gas/min, 300 ml of dry air/min, and a 300-v dc cell voltage were employed. The temperature was maintained at 250° for both the detector and the injector.

The column temperature was programmed to increase 1°/min from 160 to 250°, followed by flash heating to 280° to elute the high boiling materials from the column. The nitrogen carrier gas was adjusted to a 30-ml/min flow rate with a mass flow controller.

Sample Preparation Procedure—For statistical analysis, human blood was spiked with known amounts of the standard free bases to make 1–1000-ng/ml blood levels and equilibrated by shaking for 30 min. In the dog experiments, a 2- or 5-ml whole blood portion was withdrawn from a beagle dog, transferred immediately to a heavy-duty centrifuge tube, and weighed accurately. Two drops of 15% ethylenediaminetetracarboxylic acid disodium salt solution were added as an anticoagulant. The blood samples thus prepared were then extracted.

Ion-Pair Extraction—To the 5-ml blood sample, 4 ml of 0.2 *N* H_2SO_4 and 10 ml of ether were added. After the mixture had been shaken at room temperature for 10 min, it was centrifuged at 2000 rpm for 15 min. The ether layer was removed and discarded. Four milliliters of aqueous 3% trichloroacetic acid solution was added to the remaining blood layer, which was extracted three times using 10 ml of ether. An additional 1 ml of trichloroacetic acid was added with the ether on the second and third extractions.

For each extraction, the mixture was shaken for 30 min and centrifuged at 2000 rpm for 15 min, after which the ether layer was transferred to a separator. The combined ether extracts were washed with 10 ml of 10% NaOH and 10 ml of 10% aqueous ammonium hydroxide. After the aqueous layer had been drained, the ether layer was transferred to a conical flask and evaporated almost to dryness. The flask was further dried in a desiccator.

Ethyl Acetate Extraction—To a 5-ml blood sample in a centrifuge tube were added 1.8 ml of 0.1 *N* NaOH and 10 ml of ethyl acetate. The

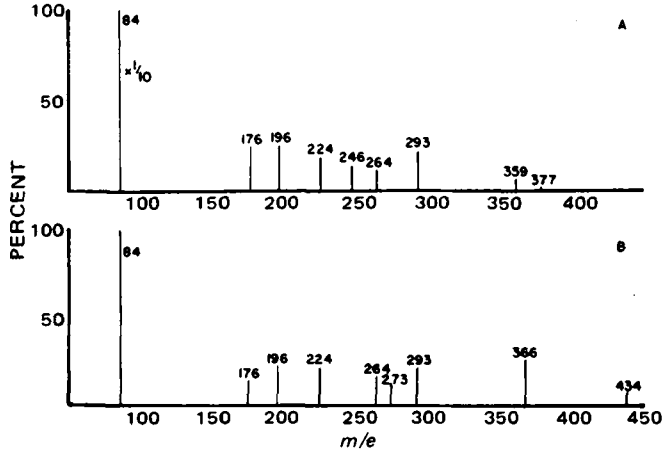


Figure 1—*Gas chromatogram–mass spectra of the free base (A) and trimethylsilane derivative (B) of mefloquine.*

[3] OV-17, Applied Science Laboratories, State College, Pa.
[4] Chromosorb W, Anspec Co., Ann Arbor, Mich.

Table II—Compound I Recovery from Spiked Blood by Ethyl Acetate Extraction followed by Electron-Capture GLC Determination

Blood Level, ng/ml	Recovery (Mean ± *SD*), %[a]
1044	68.7 ± 5.17
104.3	76.3 ± 2.77
10.5	90.1 ± 42.2
Average	78.4 ± 16.7

[a] From three replicate samples.

tube was shaken for 30 min and centrifuged at 3200 rpm for 15 min. The extraction was repeated twice. The ethyl acetate layers were transferred to a conical flask and evaporated almost to dryness. The extracts were completely dried in a desiccator.

Ether Extraction—To a 2-ml blood sample were added 0.5 ml of 0.1 *N* NaOH and 5 ml of ether. After the mixture had been shaken for 15 min at room temperature, the ether layer was separated by centrifugation at 2000 rpm for 10 min and transferred to a conical flask. The remaining layer was extracted two more times with 5 ml of ether. The combined extracts were evaporated to near dryness, and the residue was dried in a desiccator.

Trimethylsilylation—The dried residue was dissolved in ~1 ml of ether and transferred to a small conical test tube. The internal standard solution in ethyl acetate was added to the tube based on an estimated sample compound concentration. After the solvent had been evaporated carefully under a nitrogen stream, 100 μl of the trimethylsilylating reagent (200 μl if the concentration was expected to be about 500 ng/ml) was added, and the solution was left to stand for 10 min at room temperature. An appropriate volume (0.2–2.0 μl) of this solution was injected onto the GLC column.

Quantitation—A peak height ratio method was employed. The weight ratio for the calibration plot was varied by changing the mixing ratio of the standard free bases I and II in ethyl acetate solution. The mixed solution was evaporated to complete dryness and trimethylsilylated according to the described procedure. The calibration plots thus obtained showed good linearity for the flame-ionization detection over 1:5 and 5:1 weight ratios. The electron-capture detection gave a slightly concave curve in the same region; however, a 1:2–2:1 weight ratio range produced a linear electron-capture detection response. The determination limit was 10 ng/ml for I when the electron-capture detection was used in combination with ion-pair extraction, but the flame-ionization detection could detect concentrations of 100 ng/ml for both I and II.

Dog Experiment—An *in vivo* experiment was conducted using three female beagle dogs. Each dog was fasted 24 hr prior to dosing. One dog (7.75 kg) was orally dosed with one 250-mg I tablet; two dogs (13.7 and 9.0 kg) were dosed with one 250-mg II tablet (both drugs as hydrochloride salt). From the dog dosed with I, blood was withdrawn through the jugular vein at 0, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 12, 24, 72, and 144 hr, and at 2, 3, 4, 5, 6, 7, and 9 weeks after administration. The blood volume taken was 2 ml for the first 6 days and 5 ml thereafter. From the dogs dosed with II, 2 ml of blood was taken at 0, 1, 2, 3, 4, 5, 6, 8, 12, 48, and 72 hr after dosing.

RESULTS AND DISCUSSION

Since the average blood I level was expected to be lower than the II level from the same dosage, ion-pair extraction was used to obtain a better I recovery. An ethyl acetate extraction was examined as a possible procedural simplification. Tables I and II list the results for the statistical analysis of I recovery from spiked blood by ion-pair extraction and by ethyl acetate extraction, respectively. The ion-pair extraction gave higher I recovery and better reproducibility than ethyl acetate extraction; however, the standard deviation increased gradually at the lower levels. The ethyl acetate extraction was applicable to levels above 100 ng/ml. The recovery of II from the spiked blood was studied by ethyl ether extraction; a very high recovery with good reproducibility was observed for all levels examined (Table III).

In Tables I–III, the electron-capture detection and the flame-ionization detection were used for recovery determination. Electron-capture detection is highly sensitive to antimalarials that contain two trifluoromethyl groups per molecule but has a lower sensitivity to cholesterols, the major extracted blood component. The solvent peak tailing which sometimes interferes with drug peak separation in flame-ionization detection can be reduced by using low electron affinity solvents. Electron-capture detection has a relatively narrow dynamic range compared with flame-ionization detection, and detector stability and sensitivity depend

Table III—Compound II Recovery from Spiked Blood by Ether Extraction followed by Electron-Capture GLC Determination

Spiked Level, ng/ml	Recovery, %
1000	99.6
500	96.2
100	99.6
50	103.8
10	106.0
Mean ± *SD*	101.0 ± 2.8

more subtly on the detector condition than does flame-ionization detection. The detection limits for each drug were 1 ng/ml with the electron-capture detection (the limit of accurate determination is 10 ng/ml, Table I) and 100 ng/ml with the flame-ionization detection under the best condition. Thus, the electron-capture detection was used for blood levels of 10–1000 ng/ml and the flame-ionization detection was limited to levels higher than 100 ng/ml.

The I and II free bases without trimethylsilyl derivative formation gave tailing peaks on the chromatogram, which subsequently overlapped normal blood component background peaks. The trimethylsilyl derivatization produced sharper and higher peaks with shorter retention times, which helped separate the derivatives from the background peaks.

The reaction conditions used to prepare the trimethylsilyl derivatives were studied. The I and II free bases recovered from 2 or 5 ml of whole blood were completely derivatized with 100 μl of the silylating reagent at room temperature. An additional 100 μl of the reagent was used when a higher level of drug was found; the sample was further diluted so as to fall in the electron-capture detection sensitivity range. Silylation reaction time dependence was examined by injecting samples at 1, 5, 10, and 30 min after silylating reagent addition. The 1-min sample indicated about a 50% silyl derivative yield; just a trace amount of the unreacted species was found in the 5-min sample. For reaction times between 10 min and 1 day, neither a peak due to unreacted species nor measurable changes in the peak intensity were observed.

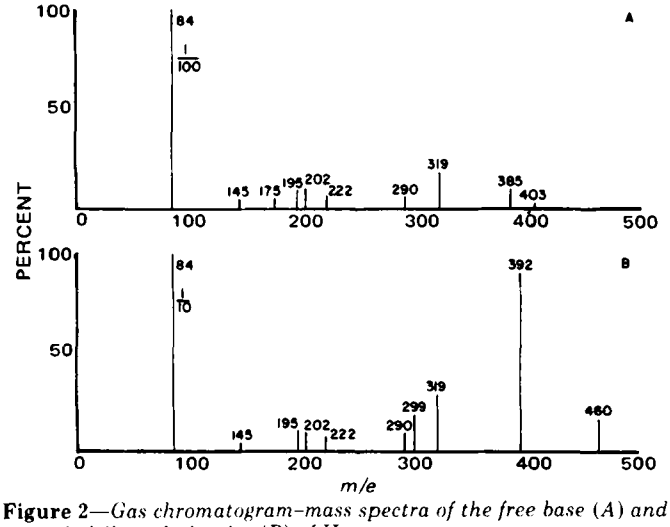


Figure 2—*Gas chromatogram–mass spectra of the free base (A) and trimethylsilane derivative (B) of II.*

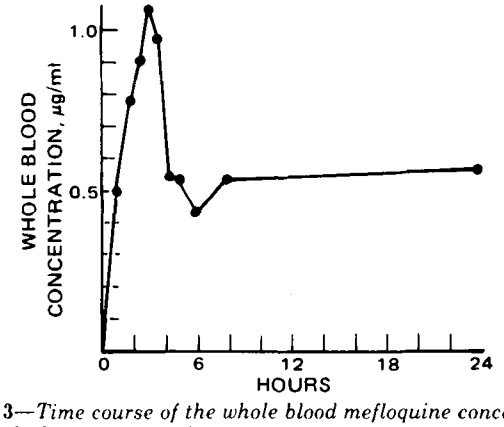
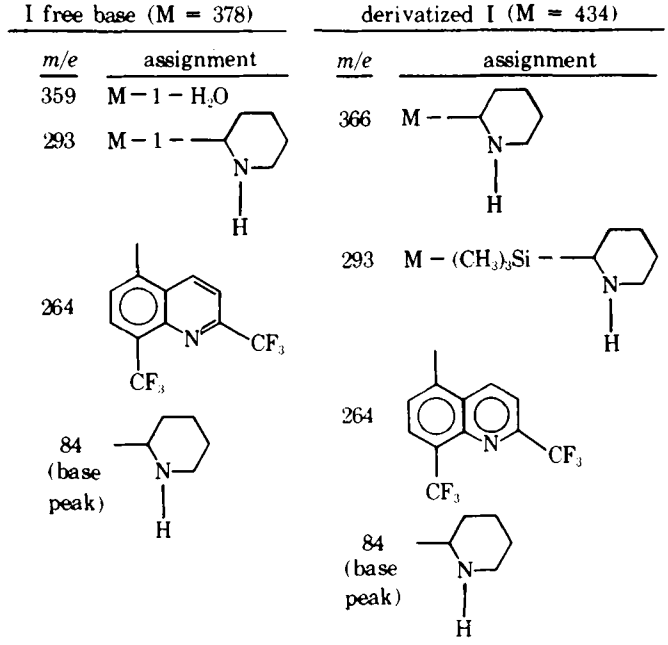
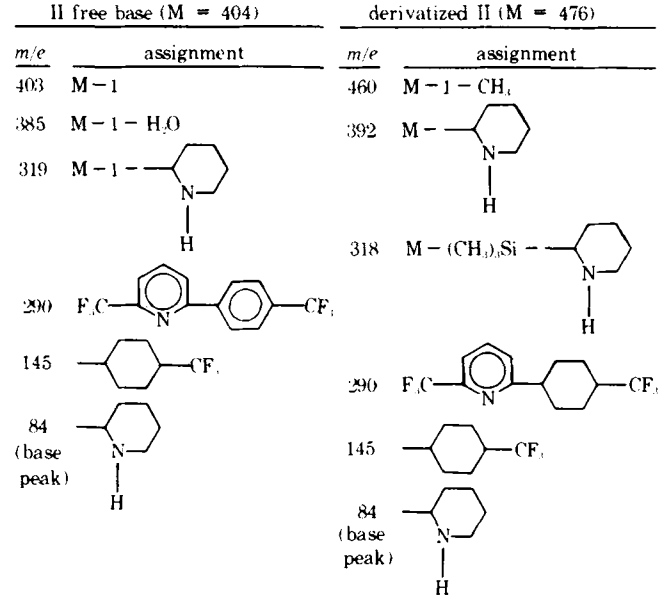


Figure 3—*Time course of the whole blood mefloquine concentration in a beagle dog given a single 250-mg tablet.*

To confirm the structure of the trimethylsilyl derivative, GLC–mass spectra of the derivatized and underivatized free bases were obtained. The spectrum for trimethylsilyl-I (Fig. 1B) indicates the formation of the $(M - 1 - CH_3)^+$ ion at *m/e* 434; the I free base had an $(M - 1)^+$ ion at *m/e* 377 (Fig. 1A). The assignments of some possible fragment ions are summarized as:



The trimethylsilyl-II spectrum (Fig. 2B) similarly indicated $(M - 1 - CH_3)^+$ ion formation at *m/e* 460, and II free base (Fig. 2A) gave the $(M - 1)^+$ ion at *m/e* 403. Some other fragment ions are shown below:



Spectra for free bases and trimethylsilyl derivatives confirmed that one trimethylsilyl group had been substituted at the hydroxy group.

In vivo experiments were carried out to examine the utility of the present method (Figs. 3–5). The flame-ionization detection was used for the samples taken in the 1st week; the electron-capture detection was used for the samples taken thereafter. Figures 3 and 4 indicate the time course of whole blood I levels for the first 24 hr and for the following 42 days, respectively. A rapid increase in the blood I level was observed up to the maximum point at 3 hr, followed by a very slow decrease to a level of 18 ng/ml at 7 weeks. No measurable level was observed in the 9th-week blood sample. A semilogarithmic plot of the data in Figs. 3 and 4 led to a I elimination half-life of 10.2 days with a 0.068 day^{-1} rate constant.

Figure 5 shows the II time course plots given as the mean of two experiments. The blood II level increased during the first 3 hr after administration, reached a maximum level which was maintained for about

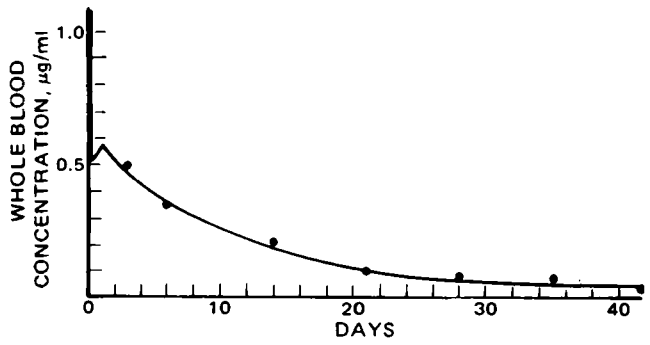


Figure 4—*Time course of the whole blood mefloquine concentration in a beagle dog given a single 250-mg tablet.*

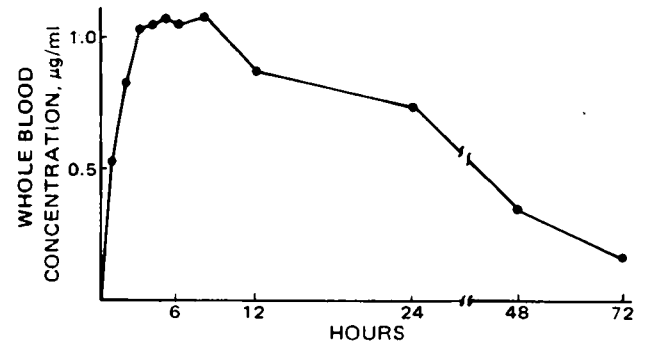


Figure 5—*Time course of the average whole blood II concentration in two beagle dogs given 250-mg tablets.*

5 hr, and then decreased slowly. From the semilogarithmic time course data plot, an elimination half-life and an elimination rate constant for II were estimated to be 24.8 hr and 0.028 hr^{-1}, respectively. The results for I and II suggested that I is about 10 times more persistent than II in its elimination from dogs.

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Powder Homogenization Using a Hammer Mill

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Abstract □ Hammer mill applicability in the comixing milling operation is discussed with reference to a 1:1000 microfine salicylic acid–sucrose binary system. The hammer mill would not serve as a mixing machine under most circumstances because of the low holdup capacity. Grinding of pure materials was preferable to mixture grinding since active ingredients could be lost during the milling operation. Remixing was always necessary following comminution of the mixture in the hammer mill. Grinding followed by remixing considerably enhanced mixture homogeneity. A large size range was produced by comminution, which resulted in the segregation of ordered units such that the final mixture could be described as a randomized ordered mixture.

Keyphrases □ Powders—homogenization using a hammer mill, sucrose–salicylic acid □ Pharmaceutical formulations—powders, homogenization using a hammer mill, sucrose–salicylic acid □ Hammer mill—powder homogenization, sucrose–salicylic acid

Many raw materials are supplied in a form requiring no further processing. However, grinding or cutting may be indicated with waxes and vegetable drugs or when agglomeration of starting materials occurs on storage. If grinding is a necessary stage in manufacturing or is required to attain a desired homogeneity, the hammer mill is frequently used for comminution.

Mixing is impossible when σ_R, the theoretical standard deviation of the fully randomized system, is equal to or greater than σ_A, the specification index (1). The σ_R value can be calculated from the sample size and the known proportions of the powders to be mixed using Lacey's equation (2):

$$\sigma_R^2 = \frac{XY}{N} \qquad \text{(Eq. 1)}$$

where X and Y are the proportions of the two ingredients and N is the number of particles in the samples. The σ_A value is calculated from the desired homogeneity of the powder mixture (3). The value of σ_R can be reduced by increasing the particle number per sample, which implies that size reduction is necessary.

Even though the hammer mill has been extensively used in the pharmaceutical industry for size reduction, limited evaluation has been carried out on the mixing performance of industrial comminution equipment. Most equipment used in comminution was ineffective for powder mixing operations because of the low holdup capacity (4). With nonflowing materials, passing a preblend through a ham-

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