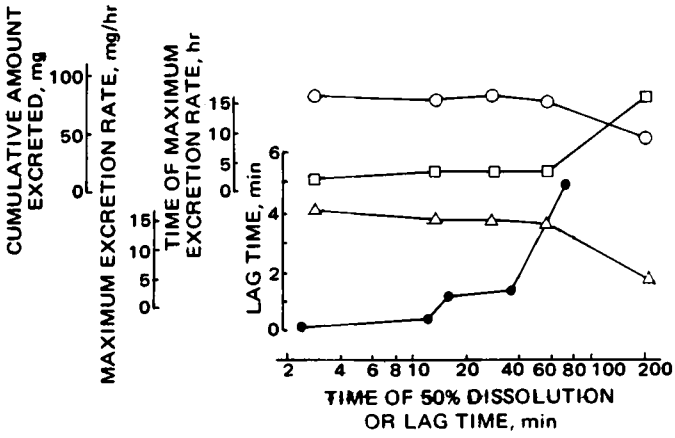

Figure 4—*Correlation of* in vitro *parameters (time of 50% dissolution and lag time) with urinary excretion* in vivo. *Key:* ●, *urinary excretion lag time;* △, *maximum urinary excretion rate;* □, *time of maximum urinary excretion rate; and* ○, *cumulative amount excreted in urine for 28 hr. These values were estimated by means of the urinary excretion of unchanged compound.*

the nonequivalent tablets. Since only one tablet was detected as nonequivalent in this study, the critical point could not be determined clearly. However, an approximate critical T_{50} point appeared to be more than 50 min. The critical value occurred surprisingly late, even though the *in vitro* rates were determined by the dissolution method that had the most vigorous agitating, dispersing, and destructive intensities among the seven methods studied.

Effect of Food on Bioavailability—The relative bioavailability of the powder and Tablet F was studied under fasting and nonfasting conditions. Table III shows the bioavailability parameters compared by the paired t test. The powder showed equivalent bioavailability with the tablets, except for Tablet F under fasting condition, and did not show any bioavailability difference between fasting and nonfasting states. Tablet F, which had poor bioavailability after fasting, became equivalent in bioavailability with the other formulations when it was administered just after the standard breakfast. Possibly, the more violent movement of the tablet in the GI tract after food might have increased drug absorption.

CONCLUSIONS

Film-coated I tablet absorption in humans is related to dissolution. The dissolution device that correlated well with bioavailability was one with a comparatively violent destructive force for the coated tablet film and high test medium agitation. The test medium pH must also be considered because dissolution of certain tablet coatings was pH dependent.

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Antitumor Agents: Structure–Activity Relationships in Tenulin Series

THOMAS G. WADDELL *x, ANNE-MARIE AUSTIN *, JANICE W. COCHRAN *, KARL G. GERHART *, IRIS H. HALL ‡, and K. H. LEE ‡

Received May 25, 1978, from the **Department of Chemistry, University of Tennessee at Chattanooga, Chattanooga, TN 37401*, and the ‡*Department of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27514.* Accepted for publication November 21, 1978.

Abstract □ Systematic structural modifications were performed on the natural sesquiterpene lactone tenulin to define those groupings essential to, or significant in, its *in vivo* antitumor activity. Accordingly, the following tenulin analogs were prepared: dihydrotenulin, 2,3-epoxytenulin, isotenulin, dihydroisotenulin, 2,3-epoxyisotenulin, and tetrahydrodeacetylisotenulin. Both the cyclopentenone and the hemiketal units in tenulin were necessary for high *in vivo* activity.

Keyphrases □ Tenulin—antineoplastic activity, structure-activity relationships, *in vivo* study □ Antineoplastic activity—tenulin, structure-activity relationships, *in vivo study*

The sesquiterpene lactone tenulin (I) is a major bitter principle in several species of the plant genus *Helenium* (Family Compositae) (1–6). High *in vivo* tenulin antitumor activity toward the Ehrlich ascites and Walker 256 carcinosarcoma screens and, to a lesser extent, toward P-388 leukemia has been demonstrated (7, 8). The tenulin cyclopentenone unit may act as a Michael acceptor in cellular enzyme essential sulfhydryl group alkylation.

Tenulin forms a Michael addition product with L-cysteine to give the zwitterionic compound II (7, 8). The adduct II, containing the cyclopentanone group, showed 84% activity loss toward the Ehrlich ascites screen, suggesting that a cyclopentenone unit is important for high activity. Low II activity also might be the result of lower solubility in lipid membranes.

In the present study, the tenulin structure was modified in ways not significantly affecting drug transport. This structure–*in vivo* antitumor activity work reveals those tenulin features necessary for high activity.

RESULTS AND DISCUSSION

Chemistry—Tenulin (I), isolated after extraction of *Helenium amarum* (6, 7), was subjected to catalytic hydrogenation conditions to give dihydrotenulin (III) (1). A new compound, 2,3-epoxytenulin (IV), was prepared in quantitative yield from tenulin upon brief treatment with cold, basic hydrogen peroxide solution. 2,3-Epoxytenulin, $C_{17}H_{22}O_6$ (by exact mass measurement), had a 194–197° melting point and IR ab-

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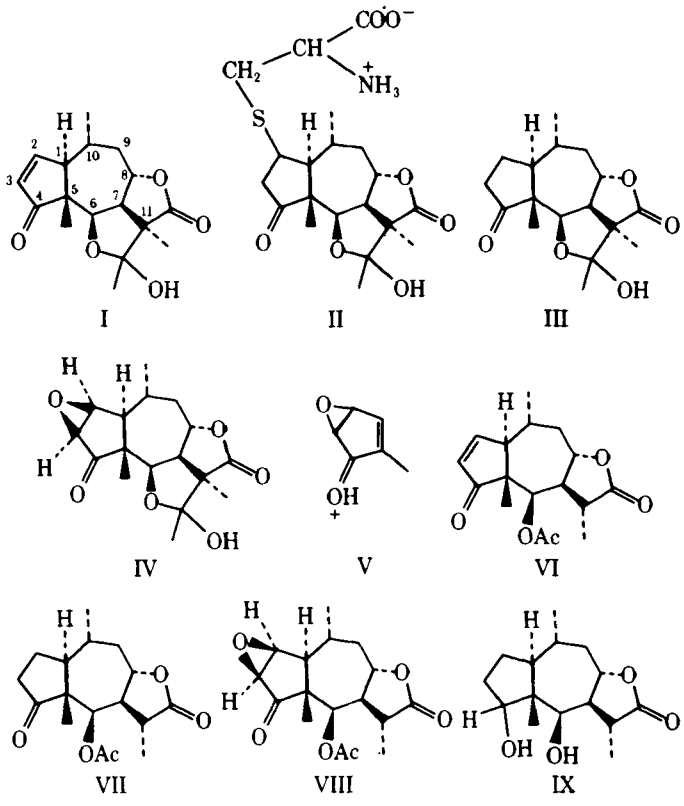

I II III IV V VI VII VIII IX

sorption at 3575 (OH), 1770 (γ-lactone), and 1750 (cyclopentanone) cm^{-1}. The UV spectrum revealed no enone absorption band at 225 nm.

The IV structure was confirmed by a high-resolution mass spectrum. Most significant of several fragment ions (see *Experimental*) were the molecular ion at *m/e* 322, an $M^+ - H_2O$ ion at *m/e* 304 indicating the presence of a hydroxyl group in IV, and the base peak at *m/e* 111 corresponding to the ion V. These and other peaks in the 2,3-epoxytenulin mass spectrum were easily interpretable in terms of Structure IV and principles recently put forth (9) regarding pseudoguaianolide mass spectra (9).

The stereochemistry of the IV 2,3-epoxide was determined by circular dichroism (Table I). The negative Cotton effect at 311 nm ($[\theta] = -6628$) corresponded to the $n \rightarrow \pi^*$ saturated ketone absorption and indicated a β-epoxide according to an inverse octant rule for epoxyketones described in the literature (10–12). This assignment for IV stereochemistry was supported by an analogous epoxidation reaction in the steroid series where 5α-androst-15-en-17-one yielded the corresponding 15β,16β-epoxy-17-ketone as the only product (13, 14).

Tenulin and 5α-androst-15-en-17-one molecular models revealed no definitive difference in steric hindrance between the molecular α- and β-faces. Therefore, an orbital explanation for the exclusive β-epoxide production from tenulin was sought. Both molecular models and circular dichroism measurements (Table I and Ref. 10) indicated that the tenulin and 5α-androst-15-en-17-one enone system was not planar but was skewed with the convex surface on the β-face (15, 16). An application of the orbital distortion technique (17) to this situation indicated that the lobes of the lowest unoccupied enone molecular orbital were distorted toward the β-face. Therefore, the attacking hydroperoxide anion should prefer to approach from this direction, as was observed experimentally.

Isotenulin (VI), prepared from tenulin by a literature method (3), yielded dihydroisotenulin (VII) (6) upon catalytic hydrogenation and 2,3-epoxyisotenulin (VIII) (6) when briefly exposed to cold, basic hydrogen peroxide solution. 2,3-Epoxyisotenulin, $C_{17}H_{22}O_6$, showed a negative Cotton effect at 312 nm (Table I) for the $n \rightarrow \pi^*$ ketone absorption, indicating that the newly introduced oxygen atom was beta. TLC showed that VIII had a higher R_f value than IV.

To prepare a derivative with a reduced ketone or lactone grouping, tenulin was reacted with sodium borohydride and with lithium aluminum hydride. In both experiments, low complex mixture yields were obtained. A sodium borohydride reduction of III produced more satisfactory results, giving, in low yield, a new compound, tetrahydrodeacetylisotenulin (IX). Compound IX, $C_{15}H_{24}O_4$, mp 161–163°, showed IR absorption at 3400 (OH) and 1755 (γ-lactone) cm^{-1}. The IX mass spectrum indicated the molecular ion at *m/e* 268 (M^+) and fragment ions at *m/e* 250 ($M^+ - H_2O$) and 232 ($M^+ - 2H_2O$), supporting the hypothesis of two hydroxyl groups. The NMR spectrum of IX (detailed under *Experimental*) was completely in accord with the assigned structure. Presumably, IX was formed in this reaction by sequential ketone reduction, retro-aldol cleavage of the hemiketal, and reductive cleavage of the resulting acetate ester.

Table I—Ketone $n \rightarrow \pi^*$ Cotton Effects for Tenulin and Derivatives

Compound	λ_{max}, nm	$[\theta]$
I	329	−3169
VI	330	−4850
IV	311	−6628
VIII	312	−9383

Biological Evaluation—Tenulin and the prepared derivatives were assayed for *in vivo* antitumor activity in Sprague–Dawley rats implanted with Walker 256 ascites carcinosarcoma cells and in DBA/2 mice implanted with P-388 lymphocytic leukemia according to published procedures (7) (Table II). Only tenulin showed significant activity in the P-388 screen. Since changes in activity are more pronounced in the sensitive Walker 256 screen, the following structure–activity relationship discussion focuses on those data.

Within the tenulin series (I, III, and IV), modification of the cyclopentenone ring by hydrogenation to III or epoxidation to IV was accompanied by decreased antitumor activity. Although less dramatic, this trend was evident also in the isotenulin series (VI–VIII) where both VII and VIII are less active than VI. These results confirmed that the tenulin and isotenulin cyclopentenone unit is needed for high *in vivo* antitumor activity. These conclusions are in accordance with those of Hall *et al.* (7) and Lee *et al.* (8), who demonstrated the tenulin cyclopentenone unit *S*-alkylating ability and related this reactivity to the action mechanism of antitumor agents bearing this enone system.

Compounds III and IV have nearly identical activities, as do VII and VIII. Furthermore, the epoxides IV and VIII, themselves potential alkylating agents, nevertheless showed decreased antitumor activity when compared to their progenitors I and VI, respectively. These observations suggest that in the tenulin–isotenulin series, the marginally active cyclopentenone epoxides are not acting significantly as *S*-alkylating agents *in vivo*.

Other systematic comparisons revealed the importance of the hemiketal unit to high antitumor activity. Within the hemiketal present–hemiketal absent pairs of I and VI, III and VII, and IV and VIII, the compound with the hemiketal intact was more active than its acetate ester counterpart. The hemiketal hydroxyl grouping may hydrogen bond the sesquiterpene antitumor agent to the cellular receptor site.

Tetrahydrodeacetylisotenulin (IX) inactivity supported the postulates regarding cyclopentenone and hemiketal importance to antitumor activity in the tenulin series.

EXPERIMENTAL[1]

***In Vivo* Tumor Screens**—*In vivo* tumor screens (Walker 256 ascites carcinosarcoma and P-388 lymphocytic leukemia) were performed according to published procedures (7). In the Walker 256 ascites carcinosarcoma screen, 10^6 tumor cells were implanted into 80 ± 10-g Sprague–Dawley male rats. In the P-388 lymphocytic leukemia screen, 10^6 cells were implanted into DBA/2 male mice (~18 g) on Day 0.

Test compounds were homogenized in 0.05% polysorbate 80–water and injected at 2.5 mg/kg/day ip for the rats and at 25 mg/kg/day ip for the mice. The day of death was recorded, and the treated/control (T/C) values for the average days survived were calculated according to the National Institutes of Health protocol 1.500 (18). Melphalan and fluorouracil were used as positive standards in these screens. Controls with 0.05% polysorbate 80–water give T/C = 100.

Isolation of Tenulin (I) from *H. amarum*[2]—A 476-g sample of *H.*

[1] Melting points were determined on a Thomas-Hoover apparatus and are corrected. IR spectra were determined with a Perkin-Elmer model 700 recording spectrophotometer. The circular dichroism curves were taken with methanol as the solvent, $c = 10^{-3}$ g/ml, using a Cary 60 instrument. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

[2] The plant material was identified as *Helenium amarum* (Raf.) H. Rock (Compositae) by Dr. Gene S. Van Horn, Department of Biology, University of Tennessee at Chattanooga. A voucher specimen (HAM-81074-FAR) is available for inspection at the Department of Chemistry, University of Tennessee at Chattanooga.

Table II—Antitumor Activity of Test Compounds [a]

Compound	Walker 256 Ascites		P-388 Lymphocytic Leukemia	
	Average Days Survived	T/C[b]	Average Days Survived	T/C[b]
I	22.16/8.33	266	13.8/10.2	135
III	24.0/16.3	147	9.5/10.2	93
IV	13.4/9.6	140	12.4/10.2	122
VI	14.6/9.6	152	11.5/10.2	113
VII	21.0/16.3	129	11.5/10.2	113
VIII	16.5/13.0	127	11.8/10.2	116
IX	15.9/15.6	102	—	—
Melphalan[c]	23.0/7.25	317	—	—
Fluorouracil[d]	—	—	19.7/10.6	186

[a] Six animals were used per group. [b] T/C ≥ 125% for minimum significant activity. [c] Wellcome Research Laboratories, Research Triangle Park, N.C. [d] Calbiochem, La Jolla, Calif.

amarum (Raf.) H. Rock leaves and stems[3] was cut into 1.27-cm pieces and extracted with chloroform by maceration for 4 days at room temperature. After decanting, the plant material was extracted twice in the same manner. The combined extracts were evaporated to dryness, leaving a dark-green tar (48.6 g). The crude extract was stirred for 30 min with 400 ml of water–ethanol (3:1) and filtered through diatomaceous earth.

The aqueous filtrate was extracted with five 100-ml portions of chloroform, and the combined organic phase was dried over anhydrous magnesium sulfate. Filtration and evaporation *in vacuo* left 19.4 g of the final extract as an amber oil. Trituration of this oil with benzene gave 6.27 g of crude I (mp 181–183°), which was recrystallized from ethanol–water, mp 186–188° [lit. (4) mp 184–186°]; IR ($CHCl_3$): 3600–3200 (OH), 1770 (γ-lactone), 1705 (cyclopentenone), and 1590 (C=C) cm^{-1}; UV (ethanol): 225 (ε 8215) nm; circular dichroism (methanol): $[\theta]_{329}$ −3169.

Anal.[4]—Calc. for $C_{17}H_{22}O_5 \cdot 0.20C_6H_6$: C, 67.91; H, 7.21. Found: C, 67.94; H, 6.90.

A direct comparison of this material with authentic I[5] established its identity. The mixed melting point of this sample was not depressed compared to authentic tenulin, the IR spectra were superimposable, and the two samples (mixed or separated) cochromatographed on TLC in numerous solvent systems. The NMR spectrum was identical to published I spectra.

Tenulin used in the experiments described in this paper showed one spot on TLC (R_f 0.50, 20% acetone in chloroform, silica gel).

Dihydrotenulin (III)—Tenulin (0.500 g) was hydrogenated in 25 ml of ethanol containing 0.20 g of 5% palladium-on-charcoal to give III (0.415 g), mp 170–171° [lit. (1) mp 172°]; IR ($CHCl_3$): 1760 (γ-lactone) and 1740 (cyclopentanone) cm^{-1}.

2,3-Epoxytenulin (IV)—To 0.203 g of I in 5 ml of methanol at 0° was added dropwise, with constant stirring, a solution containing 0.25 ml of 30% hydrogen peroxide, 0.60 ml of water, and 0.041 g of sodium carbonate. The cooled reaction mixture was stirred for 10 min, and 10 ml of distilled water was added. This aqueous solution was extracted with two 20-ml portions of chloroform, and the combined organic phase was dried (magnesium sulfate), filtered, and evaporated to dryness under reduced pressure. 2,3-Epoxytenulin was isolated as a clear glass in quantitative yield and showed a single circular spot on TLC in various solvent systems.

Efforts to crystallize this material were initially unsuccessful due to extensive decomposition. However, a crystalline sample of IV, mp 194–197°, was eventually obtained in low yield; IR ($CHCl_3$): 3575 (OH), 1770 (γ-lactone), and 1750 (cyclopentanone) cm^{-1}; circular dichroism (methanol): $[\theta]_{311}$ −6628; mass spectrum (9): *m/e* 322.1412 (M^+), calc. for $C_{17}H_{22}O_6$, 322.1416; 307 ($M^+ - CH_3$), 304 ($M^+ - H_2O$), 280, 279, 262, 261, 251, 235, 234, 233, 207, 191, 189, 163, 137, 135, 124, 123, 122, and 111 (base peak).

Isotenulin (VI)—Isotenulin was prepared in 82% yield from tenulin (3). The product VI was recrystallized from benzene–ether mixtures to give material with a melting point of 154–156° [lit. (2) mp 152–153°]; IR ($CHCl_3$): 1760 (γ-lactone), 1750 (OAc), 1705 (cyclopentenone), and 1580 (C=C) cm^{-1}; circular dichroism (methanol): $[\theta]_{330}$ −4850.

Dihydroisotenulin (VII)—Isotenulin (0.500 g) was hydrogenated in 25 ml of ethanol containing 0.231 g of 5% palladium-on-charcoal to give VII (0.441 g), mp 148–149° [lit. (6) mp 147–149°]; IR ($CHCl_3$): 1740–1760 cm^{-1}, unresolved.

2,3-Epoxyisotenulin (VIII)—2,3-Epoxyisotenulin was prepared in 74% yield from VI by the method of Lucas *et al.* (6), who obtained the product as an amorphous glass. The solid reaction product in the present case was crystallized from ethyl acetate–ether to give material with a 148–149° melting point; IR ($CHCl_3$): 1785–1755 and 1720 (shoulder) cm^{-1}; circular dichroism (methanol): $[\theta]_{312}$ −9383.

Anal.—Calc. for $C_{17}H_{22}O_6$: C, 63.35; H, 6.83. Found: C, 63.53; H, 7.01.

Tetrahydrodeacetylisotenulin (IX)—To 0.415 g of III in 10 ml of methanol was added 0.316 g of sodium borohydride in portions with constant stirring. After 45 min at room temperature, the reaction mixture was diluted to 50 ml with distilled water and extracted with three 25-ml portions of chloroform. The combined organic phase was washed once with 25 ml of water, dried (magnesium sulfate), filtered,and evaporated to dryness under reduced pressure. The crude, oily product (0.321 g) showed four components on TLC and was chromatographed on a column of silica gel (1.5 × 36 cm), eluting with chloroform followed by 20% acetone in chloroform.

From the later fractions, IX (20 mg) crystallized, mp 162–163°; IR ($CHCl_3$): 3400 (OH) and 1755 (γ-lactone) cm^{-1}; NMR ($CDCl_3$): δ 1.14 (3H, s, 5-CH_3), 1.16 (3H, d, J = 7 Hz, 10-CH_3), 1.46 (3H, d, J = 7 Hz, 11-CH_3), 3.24 (1H, m, H-11), 4.10 (1H, br, H-4), 4.18 (1H, d, J = 6 Hz, H-6), and 4.84 (1H, triplet of doublets, J = 3, 11 Hz, H-8); mass spectrum; *m/e* 268.1675 (M^+), calc. for $C_{15}H_{24}O_4$, 268.1673; 250 ($M^+ - H_2O$), 235 ($M^+ - H_2O - CH_3$), 232 ($M^+ - 2H_2O$), 224 ($M^+ - CO_2$), 208, 191, 178, 150, 136, 120, 117, 104, 103, and 102.

Anal.—Calc. for $C_{15}H_{24}O_4$: C, 67.16; H, 8.96. Found: C, 67.24; H, 9.03.

Earlier fractions from the crude reaction product chromatography yielded 3 mg of a second crystalline compound whose IR spectrum (3450, 1760, and 1720 cm^{-1}) was consistent with that expected for tetrahydroisotenulin, a possible intermediate in the production of IX.

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[4] The ability of tenulin to form strong associations with benzene is in accord with results of Ungnade and Hendley (2).

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GLC Determination of Whole Blood Antimalarial Concentrations

T. NAKAGAWA *§, T. HIGUCHI *, J. L. HASLAM ‡x, R. D. SHAFFER ‡, and D. W. MENDENHALL *

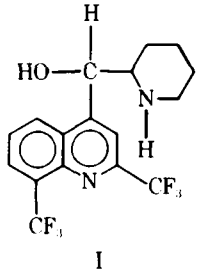
Received April 18, 1977, from the **Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, KS 66045, and ‡INTERx Research Corporation, Lawrence, KS 66044.* Accepted for publication November 21, 1978. §Present address: Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan..

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.



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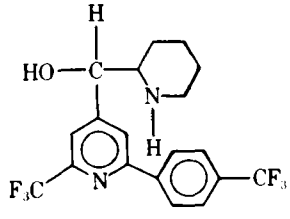

II

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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