Journal of Chromatography, 527 (1990) 115–126
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5177

Analysis of blood and urine samples from *Macaca mulata* for pyronaridine by high-performance liquid chromatography with electrochemical detection

S.A. WAGES, L.C. PATCHEN and F.C. CHURCHILL*

Control Technology Branch, Division of Parasitic Diseases, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 30333 (U.S.A.)

(First received August 18th, 1989; revised manuscript received December 14th, 1989)

SUMMARY

We describe a high-performance liquid chromatographic method with electrochemical detection for quantifying pyronaridine in rhesus monkey (*Macaca mulata*) blood and urine samples. The detection limit is 20 ng/ml at a signal-to-noise ratio of 4 in 0.5-ml samples of blood or urine. Blood analysis includes a liquid–liquid extraction and a subsequent solid-phase extraction that removes an interferent present in blood. For urine, a back-extraction is substituted for the solid-phase extraction step. The method uses an analogue of amodiaquine as internal standard, a 10-μm rigid macroporous styrene–divinylbenzene copolymer column and a mobile phase of 1% (v/v) triethylamine in methanol–water (34:66, v/v). The method was applied to samples of blood and urine from a monkey after a single intramuscular dose of pyronaridine tetraphosphate (160 mg as base).

INTRODUCTION

Pyronaridine is a naphthyridine derivative synthesized in 1970 in the People's Republic of China [1–3] and used there as an antimalarial since 1974. This drug has low mammalian toxicity [4] with minimum side-effects to humans at therapeutic doses [5–7]. Pyronaridine is effective against *Plasmodium vivax* [5–9] and *Plasmodium falciparum* [5–7,9,10] including strains of *P. falciparum* resistant to chloroquine [11,12]. A recent pharmacokinetic study using a spectrofluorimetric method measured an elimination half-life of 63 ± 5 h

0378-4347/90/$03.50

in humans for an intramuscular dose and showed that oral dosing forms had low bioavailability [13]. The effectiveness of pyronaridine in vitro at concentrations comparable to mefloquine and its apparent lack of cross-resistance to 4-aminoquinoline and quinolinemethanol antimalarials portend significant potential for widespread use in treatment of drug-resistant falciparum malaria [14]. Further clinical and pharmacokinetic evaluations of this promising drug will require selective and sensitive means for assay in blood and other body fluids.

In this report we describe a selective and sensitive high-performance liquid chromatographic (HPLC) method using oxidative electrochemical detection and an analogue of amodiaquine as an internal standard to quantify pyronaridine in blood and urine. Analysis of samples from a rhesus monkey (*Macaca mulata*) dosed with pyronaridine provides pharmacokinetic information and demonstrates the effectiveness of the method.

EXPERIMENTAL[a]

Standards

Pyronaridine tetraphosphate and pyronaridine base were obtained from Mr. Chang Chen of the Institute of Parasitic Diseases in Shanghai (People's Republic of China). The internal standard, the isobutyl analogue of desethylamodiaquine dihydrochloride {4-(7-chloro-4-quinolinyl)-2-[[(1-methylpropyl)amino]methyl]-phenol} was from Parke-Davis, Division of Warner-Lambert (Ann Arbor, MI, U.S.A.). Structures of the free bases corresponding to each of these compounds are shown in Fig. 1. Stock standard solutions were prepared by dissolving the compounds in 0.001 *M* hydrochloric acid. Working solutions were prepared by diluting these solutions in the same solvent.

Reagents and solvents

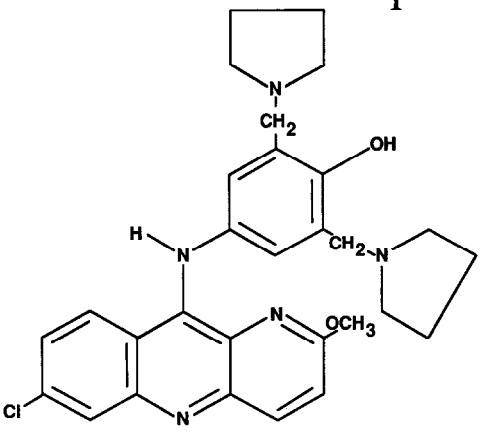
Methanol and ethyl acetate were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Methanesulfonic acid was from Eastman Kodak (Rochester, NY, U.S.A.). High-purity (HPLC grade) triethylamine (TEA) was from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other chemicals used were of reagent grade or better.

Equipment

The HPLC apparatus consisted of a Spectra-Physics Model SP8700XR pump equipped with a Model SP8500 dynamic mixer, a Rheodyne Model 7125 injector, a Hamilton PRP-1 polymer column (10 μm particle diameter; 150 mm $\times$ 4.1

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

I



II

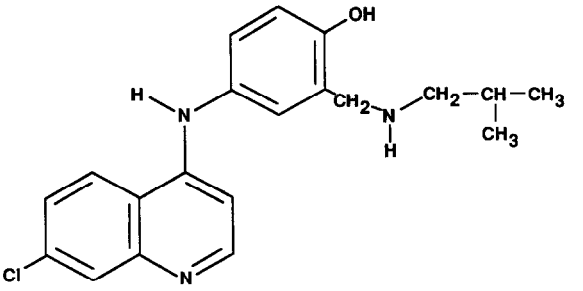


Fig. 1. Structures of pyronaridine (I) and the internal standard (II).

mm I.D.), a BioAnalytical Systems LC4B amperometric detector and a Spectra-Physics SP4270 integrator. The glassy carbon working electrode was maintained at a potential of +0.75 V versus the Ag/AgCl reference electrode.

Development of chromatographic conditions

Several HPLC columns were evaluated including C_8, C_{18} and amino bonded-phase columns and a Hamilton PRP-1 styrene–divinylbenzene copolymer column. Conditions were examined for separating pyronaridine, metabolites and internal standard candidates using aqueous mixtures containing varying concentrations of a number of buffers and organic modifiers. Final conditions for the method were developed with a mobile phase that consisted of TEA modifier in methanol–water used with the Hamilton PRP-1 column.

The HPLC mobile phase for the assay was methanol–water (34–66, v/v) containing 1% (v/v) TEA. Mobile phase pH was adjusted to 2.0 with methanesulfonic acid. The solvent was mixed off-line. Flow-rate was 0.8 ml/min,

giving a back-pressure of 16.55 MPa. The mobile phase was continuously degassed with helium during use, and all chromatography was at ambient temperature (23–28°C). The optimum oxidation potential for the assay was determined by repetitive injections of pyronaridine standard at potentials varied from +0.6 to +0.9 V (versus Ag/AgCl reference electrode) in 0.05-V increments.

Analysis of pyronaridine in blood and urine

Standards were prepared by adding 500 μl of drug-free blood, appropriate volumes of pyronaridine standard solutions to give concentrations from 0 to 2000 ng/ml, and 25 μl of internal standard solution (40 ng/μl in 0.001 *M* hydrochloric acid) to 15-ml glass centrifuge tubes. Blood samples (500 μl) and internal standard solution (25 μl) were also placed in 15-ml centrifuge tubes. Samples and standards were mixed on a vortex mixer. Aqueous 50% (v/v) K_2HPO_4 (1 ml) and ethyl acetate (6 ml) were added and the tubes capped and rotated for 30 min at 30 inversions per min to effect extraction (Glas-Col Model RD-250 rotator with a Model RD-60 rotator head; Terre Haute, IN, U.S.A.). Samples and standards were centrifuged for 10 min at 3000 *g*, and each organic layer was transferred to a clean centrifuge tube and evaporated to dryness under a gentle flow of nitrogen in a water bath held at 40°C. The concentrated extracts were reconstituted in 200 μl of 0.1 *M* hydrochloric acid in water containing 20% (v/v) methanol. Dried extracts were sonicated for 10 min to effect complete dissolution. Reconstituted extracts were each applied to a 1-ml carboxymethyl-substituted silica ion-exchange column (Analytichem International, Harbor City, CA, U.S.A.) that had been preconditioned with 2 ml of deionized water using a Vac Elut sample elution system (Analytichem International) and a moderate vacuum. A 500-μl quantity of 0.15 *M* aqueous hydrochloric acid was passed through each column, followed by elution and collection of pyronaridine and internal standard with 400 μl of methanol–1 *M* hydrochloric acid (3:1, v/v). A 20-μl quantity of each eluate was injected into the HPLC system. Samples were quantified by peak-area ratio comparisons with the standard curve.

The procedures used for initial extraction of 0.5-ml urine samples were the same as those for blood, except that the range of standards was 0–10 μg/ml and 150 μl of 40 ng/μl internal standard solution were used. After transfer of the organic layer, an aqueous acid extraction was used. Instead of sample evaporation and solid-phase extraction, a 3-ml volume of 0.1 *M* hydrochloric acid was added to the centrifuge tube followed by vortex-mixing and centrifugation at 3000 *g* for 10 min. The aqueous layer was transferred to a clean tube, and 1 ml of 10% (w/v) K_2HPO_4 and 1 ml of 0.3 *M* sodium hydroxide were added to the tube followed by 3 ml of hexane–methyl *tert.*-butyl ether (1:1, v/v). Samples were vortex-mixed for 30 s and centrifuged. The organic layer was trans-

ferred to a clean tube, dried under a gentle stream of nitrogen and reconstituted in 400 μl of mobile phase for injection into the HPLC system.

Recovery studies for extraction from blood

Standard solutions of pyronaridine and the internal standard were prepared in 0.001 *M* hydrochloric acid. Concentrations were accurately known. Triplicate 500-μl quantities of whole blood were fortified with various concentrations of pyronaridine and internal standard. The extracts were injected and compared with the unextracted solutions after appropriate adjustments for aliquoting in the extraction procedure. The same procedure was used for studies of the recovery of pyronaridine from urine.

Storage stability study

Drug-free whole blood was spiked with pyronaridine at three different concentrations. An aliquot (10 ml) of each was stored in the refrigerator at 4 °C. A second portion of the spiked whole blood was further subdivided and frozen at −20 °C in individual 1-ml plastic centrifuge tubes for subsequent analysis. Samples were run in duplicate on day 1 and weeks 1, 3, 4, 8, 12, 16 and 20 along with standards that were prepared on the day of the analysis.

Dosing of rhesus monkey

A rhesus monkey weighing 9 kg was injected intramuscularly with pyronaridine tetraphosphate (160 mg per week as base) for three consecutive weeks. Following the third dose, venepuncture samples were taken to establish the concentration profile over time for pyronaridine and any metabolites. Samples were taken over a period of 28 days.

The same rhesus monkey was later given pyronaridine orally. For this experiment, the pyronaridine salt was dissolved in 10 ml of water and administered under light anesthesia (ketamine) via a naso-gastric tube. This study was initiated four months after administering the final intramuscular dose, with a whole blood sample taken before the first oral dose to verify essentially complete elimination of the drug (pyronaridine concentration in blood was below the detection limit of the method). According to the protocol, the monkey was given the same weekly dosage orally that was administered intramuscularly (160 mg base). When no pyronaridine was detected in the whole blood after the first or second dose, the dosage was increased on the third week to 60 mg/kg (570 mg pyronaridine as base). No apparent side-effects were noted in the monkey at the higher dose. Following the dose, venepuncture samples were taken at 4 and 30 h and concentrations measured.

Urine was collected for the first 24 h following the second and third weekly doses of pyronaridine for the intramuscular injection study protocol and following all three doses for the oral protocol to assay for the presence of the

parent drug and any metabolites present. Also, urine was collected seven days after the week 3 oral dose of drug.

RESULTS AND DISCUSSION

Characterization of the method

C_8 and C_{18} columns gave broad, tailing peaks under a variety of HPLC conditions. The amino-bonded column showed poor selectivity for pyronaridine and for related compounds such as amodiaquine analogues investigated as internal standard candidates. We did not believe that the column would effectively separate pyronaridine and any metabolites that might be present. The PRP-1 column was found to give sharp, symmetrical peaks and good selectivity with methanol–water mobile phases containing TEA and acidified with methanesulfonic acid. Pyronaridine and the internal standard were extracted initially from whole blood with the same method used for extracting urine, which is similar to that used earlier for amodiaquine [15]. The chromatogram resulting from assay of drug-free plasma revealed an unknown compound with the same retention time as pyronaridine under the conditions established for separating pyronaridine and the internal standard. Several options existed to resolve this interference. The chromatographic solvent system could be adjusted to resolve the two peaks, the extraction procedure could be modified or the mode of detection could be changed to improve selectivity. Altering the

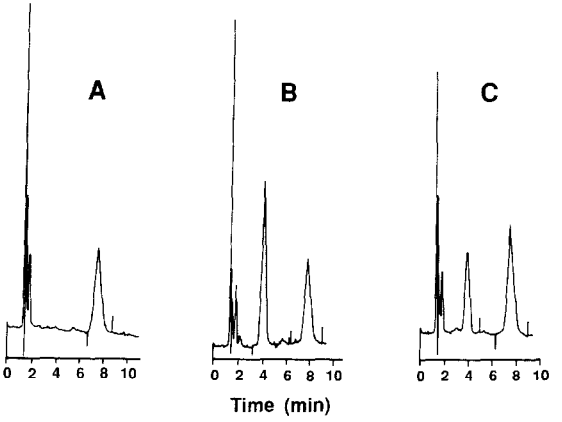


Fig. 2. Chromatograms from application of the method to (A) an extract of a 0.5-ml drug-free rhesus monkey blood containing 25 μl of 40 ng/ml internal standard solution alone, (B) an extract of a 0.5-ml drug-free blood standard containing 754 ng/ml pyronaridine and (C) an extract of a 0.5-ml blood sample from a rhesus monkey taken 24 h after a 160 mg (as base) intramuscular dose of pyronaridine tetraphosphate and found to contain 290 ng/ml pyronaridine. An extract of a predose blood sample showed no peaks at the retention times corresponding with elution of pyronaridine or the internal standard.

TABLE I

TYPICAL PYRONARIDINE STANDARD CURVE

Pyronaridine added (ng/ml)	Pyronaridine/internal standard peak-area ratio	Pyronaridine calculated (ng/ml)
2260	2.2028	2246.0
	2.2174	2261.0
753	0.7636	766.8
	0.7712	774.7
251	0.2754	265.1
	0.2571	246.3
84	0.1014	86.27
	0.1009	85.75
0	0.0000	4.09

TABLE II

BETWEEN-SAMPLE AND BETWEEN-INJECTION REPRODUCIBILITY FOR PYRONARIDINE IN 0.5-ml SAMPLES OF WHOLE BLOOD AND URINE BY HPLC ANALYSIS

Sample	Pyronaridine concentration (ng/ml)	Reproducibility (%)	
		Between-sample ($n=5$)	Between-injection ($n=5$)
Blood	999	3.9	3.6
	333	6.5	4.3
	20	12.7	13.0
Urine	2600	2.2	2.0
	296	8.1	4.1

extraction procedure was found to be the best method for eliminating the interfering compound.

Modification of the sample preparation procedure involved adding a solid-phase extraction to selectively retain the interfering compound. A cation-exchange column was used. With a series of solvents, it was possible to elute pyronaridine along with the internal standard whilst retaining the unknown interferent. Injection of a 20-μl aliquot of the eluate into the HPLC column allowed quantitation of pyronaridine.

Examples of chromatograms resulting from the HPLC assay of pyronaridine in whole blood are shown in Fig. 2. The voltammetric curve plateaued at +0.75 V (versus the Ag/AgCl reference electrode), which was chosen as the analytical potential. The response was linear over the concentration range examined (Table I). The linear least-squares relationship for pyronaridine standardization was $y=9.73\cdot10^{-4}x+0.01746$; $r^2=0.9998$ ($n=5$). The detection limit for

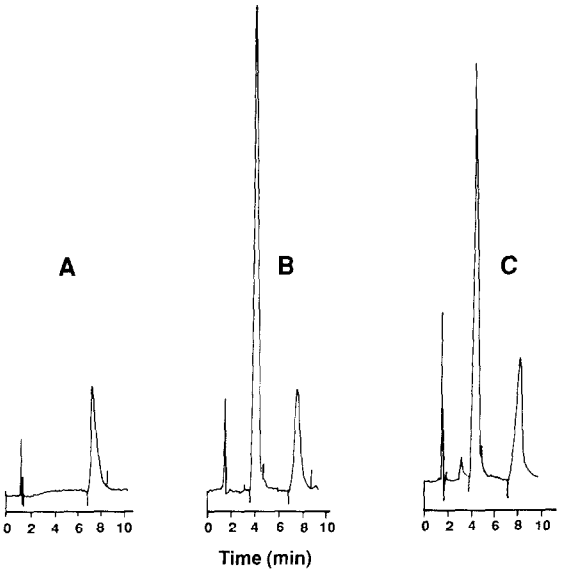


Fig. 3. Chromatograms from application of the method to (A) an extract of a 0.5-ml drug-free urine containing added internal standard (150 μl of 40 ng/ml solution) alone, (B) an extract of a 0.5-ml drug-free urine standard containing 8 μg/ml pyronaridine and (C) an extract of a 24-h urine collection sample (0.5 ml) from a rhesus monkey after a 160-mg (as base) intramuscular dose of pyronaridine tetraphosphate and found to contain 5.72 μg/ml pyronaridine. An extract of a pre-dose urine sample showed no peaks at the retention times corresponding with elution of pyronaridine or the internal standard. The peak at 3.0 min may be due to a metabolite.

pyronaridine in blood was 20 ng/ml for a 0.5-ml sample (signal-to-noise ratio of 4). The limit of determination was also 20 ng/ml (Table II). The precision indicated by duplicate standards for each point of the standard curve (Table I) was demonstrated at the 333 and 999 ng/ml levels for $n=5$ (Table II). Recoveries of pyronaridine from whole blood averaged 60% within the range of concentrations represented by the standard curve.

HPLC profiles of urine extracts of standards and samples are shown in Fig. 3. Response was linear for the concentration range 0.0–10.0 μg/ml ($y=6.64\cdot10^{-4}x-0.0204$; $r^2=0.9987$, $n=5$). The sensitivity is comparable to that for the blood determination, and recoveries averaged 73 and 78% at 2.6 and 0.30 μg/ml, respectively.

Storage stability of blood samples

The findings obtained from the storage stability study showed a slow decrease in the pyronaridine concentrations in both the refrigerated and frozen whole blood samples (Table III). Losses in extractable quinacrine, another nitrogen heterocyclic antiparasitic drug, were similarly shown upon sample

TABLE III

STORAGE STABILITY OF PYRONARIDINE IN REFRIGERATED (4°C) AND FROZEN (−20°C) WHOLE BLOOD SAMPLES

Time (weeks)	Concentration of pyronaridine[a]		
	900 ng/ml	450 ng/ml	150 ng/ml
Refrigerated			
Day 1	865	455	151
1	885	440	144
3	830	437	150
4	883	425	149
8	744	356	131
12	540	281	111
16	511	202	100
20	524	257	101
Frozen			
Day 1	887	442	152
1	896	458	130
3	802	413	129
4	734	372	125
8	673	305	133
12	542	300	90
16	424	267	102
20	507	273	109

[a]Nominal, from preparation for standards by weight.

storage [16]. The refrigerated pyronaridine samples showed little loss in the week 4 samples, but by week 8 the concentration in these samples had decreased by 13–21%. The concentration steadily decreased over the next three months. The same trend is seen for frozen whole blood, except that a significant decrease in pyronaridine is seen earlier (week 4). By week 4 the frozen pyronaridine-spiked whole blood had decreased by approximately 16%. No concentration dependency apparently exists for the pyronaridine losses. No extra peaks were observed in the chromatograms. It is possible that this pyronaridine loss is due to irreversible binding to blood constituents. All samples tested in the rhesus monkey study were refrigerated at 4°C and were extracted from the whole blood by week 3 following the blood sampling.

Rhesus monkey study

The monkey received an intramuscular dose of pyronaridine tetraphosphate (160 mg/week as base) for three consecutive weeks. Blood samples taken at intervals for 28 days following the final dose gave the pyronaridine concentrations shown in Table IV. As seen in Fig. 4, the concentration was around 2000

TABLE IV

CONCENTRATION OF PYRONARIDINE IN WHOLE BLOOD OF RHESUS MONKEY DOSED INTRAMUSCULARLY

Time after week 3 dose		Concentration (ng/ml)
h	days	
0.5		1891
1.0		1983
2.0		1319
3.0		1383
4.0		1171
8.0		701
	1	290
	2	170
	3	155
	7	71
	14	27
	21	<20
	28	<20

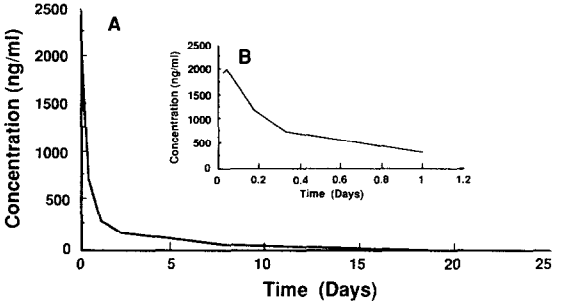


Fig. 4. Pyronaridine concentrations in whole blood of a rhesus monkey after a single intramuscular dose of pyronaridine phosphate (160 mg as base). Trace A shows the full data set, while trace B is restricted to the first 24 h after dosing.

ng/ml (1983 ng/ml, Table IV) 1 h following the dose and then declined rapidly. The concentration of pyronaridine was below the detection limit by day 21. The terminal elimination half-life for pyronaridine, determined graphically and using a non-linear curve-fitting program (PCNONLIN) [17] run on an IBM-PCAT computer, was 64 h.

The concentration of pyronaridine in blood of the rhesus monkey after the oral dose was considerably lower than for the intramuscular dose. The blood samples taken on weeks 1 and 2, corresponding to the lower oral dose (160 mg), contained no detectable pyronaridine. The samples collected on week 3, 4 h after administering 540 mg (60 mg/kg), contained a concentration of 88

ng/ml, while the sample collected at 30 h contained 142 ng/ml. Due to the low pyronaridine levels no further blood samples were drawn.

Urine collected during both the intramuscular and oral drug administration was assayed (Table V). Compounds were present in the drug-free urine that appear in the chromatogram but do not interfere chromatographically with pyronaridine or the internal standard. The urine collected during the 24-h period after the week 2 intramuscular dosing contained approximately twenty times the pyronaridine concentrations found at the corresponding time after the same dose given orally. Increasing the oral dose to 540 mg in week 3 produced no ill effect in the monkey but resulted in a pyronaridine concentration in urine only slightly higher than for week 2. The solution of pyronaridine tetraphosphate in water is therefore not readily bioavailable. An intramuscular dose given in week 4 of the oral dosing experiment gave expectedly high urinary pyronaridine levels. Chromatograms of urine extracts from the pyronaridine-dosed rhesus monkey show a peak at a retention time of 3 min, possibly due to a metabolite, in addition to pyronaridine and internal standard peaks (Fig. 3).

It is important to know the extent to which pyronaridine is metabolized in mammals. The data obtained from the whole blood and urine analysis indicate that metabolites of pyronaridine are absent or present in very small amounts in the whole blood. Evidence for at least one metabolite was found in urine extract chromatograms. Small amounts of this metabolite were found in urine collected following the intramuscular dosing as well as in urine that was collected seven days after the 60 mg/kg oral dose. Assuming the parent drug and metabolite have the same reactivities to electrochemical oxidation, the concentration of this compound in the urine is estimated to be between 100 and 200 ng/ml.

TABLE V

AMOUNT OF PYRONARIDINE IN URINE OF RHESUS MONKEY

Time	Pyronaridine dose (mg)	Pyronaridine concentration (ng/ml)
Intramuscular		
Week 2	160	9230
Week 3	160	5720
Oral		
Week 1	160	173
Week 2	160	450
Week 3	540	642
Week 3/Day 7	–	230
Week 4/Day 1[a]	160	6420
Week 4/Day 2	–	156

[a]Intramuscular dosing.

In summary, this work describes a sensitive and selective HPLC method using electrochemical detection for monitoring pyronaridine concentrations in whole blood obtained from monkeys. The method will be applied in support of clinical and pharmacokinetic studies to evaluate the prospects for use of this promising drug outside China. The potential for widespread use of pyronaridine against chloroquine-resistant falciparum malaria is of primary interest.

ACKNOWLEDGEMENTS

This work was done while the first author held a National Research Council–Centers for Disease Control Research Associateship. We thank Valerie Filipski for her help with the rhesus monkey dosing studies. We are indebted to Mr. Chang Chen, Institute for Parasitic Diseases, CAPM, for valuable discussions during his three-month tenure as a Guest Researcher in our laboratory at the Centers for Disease Control under the auspices of the Protocol between the Department of Health and Human Services of the United States of America and the Ministry of Public Health of the People's Republic of China for Cooperation in the Science and Technology of Medicine and Public Health.

REFERENCES

1 X.Y. Zheng, Y. Xia, F.H. Gao, H.Z. Guo and C. Chen, Acta Pharm. Sinica, 14 (1979) 736.
2 C. Chen, Pharm. Ind., 9 (1981) 12.
3 X.Y. Zheng, C. Chen, F.H. Gao, P. Zhu and H.Z. Guo, Acta Pharm. Sinica, 17 (1982) 118.
4 New Drug Group of Former Department of Malaria, Acta Pharm. Sinica, 15 (1980) 630.
5 Y.X. Xu, D.Q. Liu, Y.C. Wang, F.Y. Zhu and L.G. Li, Natl. Med. J. China, 62 (1982) 686.
6 Y.X. Xu, Y.C. Wang, D.Q. Liu, J.L. Sun, Z.C. Gu, X.Y. Zheng and H.Z. Guo, Chin. J. Intern. Med., 21 (1982) 655.
7 Z.S. Huang, D.Q. Liu, Y.C. Wang, C.F. Liu, J.X. Wang, M.J. Wang, Y.X. Xu, X.Y. Zheng and Q.Q. Chen, Natl. Med. J. China, 65 (1985) 366.
8 W.M. Kang, R.H. Yang, R.Z. Huang, H.Q. Xu, C. Xie, Q.H. Liu, J.S. Ye and Y.H. Xi, J. Parasitol. Parasitic Dis., 2 (1984) 263.
9 Pyronaridine Research Cooperative Group, Chin. J. Intern. Med., 24 (1985) 646.
10 Q.L. Weng and Y.C. Liu, New Drugs Clin. Rem., 6 (1987) 246.
11 J. LaPierre, Nouv. Presse Med., 11 (1982) 673.
12 X.Z. Cai, X.J. Pang and W.J. Cheng, Chin. J. Parasitol. Parasitic Dis., 5 (1987) 228.
13 Z. Feng, Z.F. Wu, C.Y. Wang and N.X. Jiang, Acta Pharmacol. Sinica, 8 (1987) 543.
14 G.E. Childs, B. Häusler, W. Milhous, C. Chen, T. Wimonwattrawatee, N. Pooyindee and E.F. Boudreau, Am. J. Trop. Med. Hyg., 38 (1988) 24.
15 F.C. Churchill, L.C. Patchen, C.C. Campbell, I.K. Schwartz, P. Nguyen-Dinh and C.M. Dickinson, J. Chromatogr., 36 (1985) 53.
16 S. Björkman and L.E. Elisson, J. Chromatogr., 420 (1987) 341.
17 Statistical Consultants, Am. Stat., 40 (1986) 52.