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ANALYSIS OF INVESTIGATIONAL DRUGS IN BIOLOGICAL FLUIDS METHOD DEVELOPMENT AND ROUTINE ASSAY

ANNUAL REPORT

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June 3, 1991

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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This report describes the status of work under contract DAMD17-86-C-6150 at the end of the reporting period, March 15, 1990 to March 14, 1991. Analytical methodologies were under development for the determination of the concentrations of the anti-viral drug, ribavirin, and WR 249,992 in plasma; the anti-malarial drug, ß-arteether, and dihydroginghaosu in biological fluids, the anti-malarial drug, halofantrine, and its metabolite, WR 178,460, in blood and plasma; the anti-leishmanial compound, WR 6026 in blood and plasma and the 4-hydroxymethyl metabolite of WR 6026 (WR 211,789) in biological fluids; mefloquine in blood and the carboxyl metabolite of mefloquine, WR 160,972, in biological fluids. Study reports were submitted for the ribavirin and halofantrine methods. Routine assays for nine studies were underway during the fifth year of the contract for analysis of plasma and blood for halofantrine and WR 178,460 ; plasma and infusates for pyridostigmine; blood for mefloquine; and plasma for WR 6026 and WR 211,789. Analysis reports were submitted for six studies.

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TABLE OF CONTENTS

Foreword ---------- 1

Introduction ---------- 3

Discussion ---------- 3

- Table 1: Previous Related Submittals ---------- 4
- Table 2: Chromatographic Systems Committed to Development ---------- 7
- Table 3: Chromatographic Systems Committed to Routine Analysis – 8

Documentation ---------- 9

- Submittals Related to Contract DAMD17-86-C-6150 ---------- 9
- Publications ---------- 10

Status of Accomplishments ---------- 10

- Ribavirin ---------- 10
- ß-Arteether and Dihydroginghaosu ---------- 10
- Halofantrine ---------- 11
- Pyridostigmine ---------- 12
- WR 6026 & WR 211,789 ---------- 12
- Mefloquine and WR 160,972 ---------- 13
- Artelinic Acid ---------- 13

Tests ---------- 15

INTRODUCTION

The laboratory has contracted (contract number DAMD17-86-C-6150) with the US military establishment to develop and use analytical methods for detection and quantitation of candidate chemical warfare antidotes, radioprotectants and anti-infectious disease drugs to support pharmacokinetic and bioavailability studies for the purpose of new drug development. The laboratory during the reporting period March 15, 1990 to March 14, 1991 completed development of two methodologies (ribavirin and WR 249,992 in plasma and halofantrine and WR 178,460 in plasma and blood), continued development of two methodologies (4-hydroxy metabolite of WR 6026 in biological fluids; and WR 160,972 (a mefloquine metabolite) in biological fluids), halted development of one methodology (b-arteether and dihydroginghaosu in biological fluid), began development of one methodology (artelinic acid in biological fluid) and ran routine analyses on 3276 specimens in support of six studies.

Reporting instructions required the completion of four quarterly project status and funds expenditure reports, one annual report, two study reports, and six routine analysis reports, as detailed in "Documentation, Submittals Related to Contract DAMD17-86-C-6150 for 1990-1991." Previous related submittals have been issued for contract DAMD17-86-C-6150, as detailed in Table 1.

No great difficulties are foreseen in completing methods development or performing routine analyses for the drugs and studies currently targeted. The technical approach that is being used in the development process is to adapt high-performance liquid chromatographic methods to the analysis of specimens for each of the compounds of interest.

DISCUSSION

The technical work performed during the fifth year of the contract involved using accepted scientific procedures combined with the equipment and facilities of the University to obtain the data and to proceed with the development of methodologies as detailed elsewhere in this report. Accepted scientific procedures including normal and reversed phase high-performance liquid chromatographic methods, post column derivatization, and protein precipitation, extraction, and cartridge elution sample clean up procedures were employed in development and routine work. Tables 2 and 3 list the five chromatographic systems committed to development of methodologies, each complete with a Waters Intelligent Sample Processor (WISP) and a Hewlett Packard Integrator 3392A, and the nine chromatographic systems committed to the analysis of submitted specimens.

In addition, the development laboratory is equipped with three general purpose fume hoods, clean room facilities, three lockable explosion proof refrigerators, three -20°C and two -80°C freezers, one drug cabinet, two fire proof solvent cabinets and a

TABLE 1: PREVIOUS RELATED SUBMITTALS

Report Type	Report Number	Period Covered by or Drug and Specimen Type of Report	Date of Report
Annual	1	1986-1987	Apr. 13, 87
Annual (Revised)	1		Jun. 11, 87
Annual	2	1987-1988	Apr. 12, 88
Annual (Revised)	2		May 20, 88
Annual	3	1988-1989	Apr. 17, 89
Annual (Revised)	3		May 30, 89
Project Status	1	March 15-June 14, 1986	Jun. 30, 86
Project Status	2	June 15-September 14, 1986	Sep. 30, 86
Project Status	3	September 15,-December 14, 1986	Jan. 27, 87
Project Status	4	December 15, 1986-March 14, 1987	Mar. 31, 87
Project Status	5	March 15-June 14, 1987	Jun. 26, 87
Project Status	6	June 15-September 14, 1987	Sep. 30, 87
Project Status	7	September 15,-December 14, 1987	Dec. 23, 87
Project Status	8	December 15, 1987-March 14, 1988	Mar. 30, 88
Project Status	9	March 15-June 14, 1988	Jun. 30, 88
Project Status	10	June 15-September 14, 1988	Oct. 4, 88
Project Status	11	September 15,-December 14, 1988	Jan. 4, 89
Project Status	12	December 15, 1988-March 14, 1989	Mar. 30, 89
Project Status	13	March 15-June 14, 1989	Jul. 12, 89
Project Status	14	June 15-September 14, 1989	Oct. 2, 89
Project Status	15	September 15,-December 14, 1989	Jan. 2, 90
Project Status	16	December 15, 1989-March 14, 1990	Apr. 2, 90
Study	6	Mefloquine in plasma	May 4, 87
Study	6B		Oct. 19, 87
Study	6C		Jan. 8, 88
Study	7	Pyridostigmine in urine	May 12, 87
Study	7B		Jan. 12, 88
Study	8	Physostigmine in plasma	Oct. 20, 87
Study	8B		July 28, 88
Study	8C		Sept. 23, 88

TABLE 1: PREVIOUS RELATED SUBMITTALS (CONTINUED)

Report Type	Report Number	Drug and Specimen Type	Date of Report
Study	9	Physostigmine in plasma	May 27, 88
Study	9B		Sept. 12, 88
Study	10	WR 6026 in plasma and blood	Jan. 20, 89
Study	10B		Apr. 7, 89
Study	10C		Sep. 14, 89
Study	11	WR 2721 in plasma	Dec. 29, 88
Study	11B		Sep. 28, 89
Study	12	WR 3689 in plasma	Dec. 29, 88
Study	12B		Nov. 14, 89
Study	13	WR 238,605 in plasma	Mar. 29, 89
Study	13B		Nov. 17, 89
Study	14	Mefloquine in plasma	Mar. 13, 89
Study	14B		Aug. 29, 89
Analysis	AY86-1A	Halofantrine in plasma	Sep. 29, 86
Analysis	AY86-1B		Jun. 11, 87
Analysis	AY86-1C		Aug. 13, 87
Analysis	AY86-1D		Oct. 23, 87
Analysis	AY86-2A	WR 6026 in plasma	Oct. 29, 86
Analysis	AY86-2B		Feb. 12, 87
Analysis	AY86-2C		Apr. 23, 87
Analysis	AY86-2D		Jun. 24, 87
Analysis	AY86-3	Pyridostigmine in urine	Jan 27, 87
Analysis	Pyr/U 86-3B (revision of AY86-3)		Feb. 3, 88
Analysis	Mef/P 87-1	Mefloquine in plasma	Sep. 16, 87
Analysis	Mef/P 87-1B		Feb. 25, 88
Analysis	Pyr/PU 87-2	Pyridostigmine in plasma and urine	Jan 27, 88
Analysis	Pyr/PU 87-2B		Feb. 24, 88
Analysis	Pyr/P 88-1	Pyridostigmine in beagle dog plasma	Mar. 29, 88
Analysis	Pyr/P 88-1B		Apr. 26, 1988

TABLE 1: PREVIOUS RELATED SUBMITTALS (CONTINUED)

Report Type	Report Number	Drug and Specimen Type	Date of Report
Analysis	Pyr/P 88-2	Pyridostigmine in plasma	May 5, 1988
Analysis	Pyr/P 88-2B		June 13, 1988
Analysis	Pyr/P 88-2C		Aug. 3, 1988
Analysis	Pyr/P 88-3	Pyridostigmine in plasma	Aug. 2, 1988
Analysis	Phy/P 88-5	Physostigmine in beagle dog plasma	Aug. 26, 1988
Analysis	Phy/P 88-5B		Oct. 19, 1988
Analysis	Phy/P 88-6	Physostigmine in rhesus plasma	Sept. 15, 1988
Analysis	Phy/P 88-6B		Oct. 19, 1988
Analysis	WR6/B 88-7	WR 6026 in blood	Apr. 21, 1989
Analysis	Phy/rP 88-8	Physostigmine in rat plasma	Sep. 14, 1988
Analysis	Phy/rPr 88-9	Physostigmine in rat perfusate	Sep. 14, 1988
Analysis	Phy/mP 88-10	Physostigmine in monkey plasma	May 5, 1988
Analysis	Mef/P 88-11	Mefloquine in plasma	Dec. 8, 1988
Analysis	Mef/P 88-11B		Jan. 31, 1990
Analysis	WR5/P 89-1	WR 238,605 in plasma	Apr. 13, 1989
Analysis	Pyr/P 89-2A	Pyridostigmine in plasma	May. 12, 1989
Analysis	Pyr/P 89-2B		Nov. 28, 1989
Analysis	Pyr/P 89-3A	Pyridostigmine in plasma	May. 16, 1989
Analysis	Pyr/P 89-3B		Nov. 30, 1989
Analysis	WR5/BP 89-4A	WR 238,605 in plasma and blood	Jun. 1, 1989
Analysis	WR5/BP 89-4B		Dec. 5, 1989
Analysis	WR5/BP 89-5	WR 238,605 in plasma and blood	Aug. 25, 1989
Analysis	Phr/P 89-6A	Physostigmine in plasma	Sep. 26, 1989
Analysis	Phr/P 89-6B		Jan. 18, 1990

60 sq. ft. solvent room. The routine analysis laboratory is equipped with two fume hoods, two explosion proof refrigerators, two -20°C freezers, one -80°C freezer, and one fire proof solvent cabinet. These and all the usual laboratory equipment (e.g., balances, pH meters, incubators, centrifuges, pipetters, etc.) necessary for preparing biological samples have been used in methods development and routine assays. During the first year of the contract, two refrigerated WISPs for the WR 2721 and WR 3689 assays, two Kratos post column reactors for the physostigmine assay, and

two refrigerators for the South San Francisco laboratory were purchased. During the second year of the contract, two additional refrigeration units for WISP systems were purchased. During the third year of the contract, two RF-535 Shimadzu fluorescence detectors were purchased. During the fourth year of the contract, a biohazard hood and a -80°C freezer were purchased. During the fifth year of the contract, no major purchases were made. In addition, since the first year of the contract, a Varian model 5000 gradient pump, two Varian model 8500 pumps, a Perkin Elmer 203 fluorescent detector, and two Shimadzu RF-530 fluorescent detectors were replaced with an LDC model CM 4000 gradient pump, two Rainin HPLX pumps, an Hitachi model 1000 fluorescent detector, and two Shimadzu RF-535 fluorescent detectors, respectively. An ESA electrochemical detector, the two Kratos reactors and a GC-MS were purchased with funds unrelated to the DAMD17-86-C-6150 contract, but this equipment can be made available, if needed, with contract funding.

The facilities dedicated for use under this contract encompass rooms 822 and 824 of the Medical Sciences Building and rooms 1257 and 1258 of the Health Science East Building located at the University of California, San Francisco and the off campus laboratory at 296 Lawrence Dr., South San Francisco. The facilities occupy 3900 sq. ft. of space. Additional facilities at the South San Francisco laboratory will be available during the fifth year of the contract.

Data for an analysis is obtained by comparison of the results for a sample with the results for a series of standard curve samples. The standard curve is constructed by finding the best fit straight line with linear regression analysis of the peak height ratio of the drug to an internal standard versus the spiked concentration of prepared

TABLE 2: CHROMATOGRAPHIC SYSTEMS COMMITTED TO DEVELOPMENT

System	Pump	Detector
1	LDC CM 4000 gradient pump	Perkin Elmer 650-10S fluorescent detector
2	Beckman 110A	Perkin Elmer 204-A fluorescent detector
3	Waters 6000	Kratos Spectroflow 773 variable wavelength UV detector
4	Waters 6000	Kratos Spectroflow 773 variable wavelength UV detector
5	Beckman 100	Bioanalytical LC-4B electrochemical detector

TABLE 3: CHROMATOGRAPHIC SYSTEMS COMMITTED TO ROUTINE ANALYSIS

System	Pump	Detector	Attachment
1	Beckman 110A	Kratos Spectroflow 773 variable wavelength UV detector	WISP 710B and Integrator
2	Beckman 110A	Kratos Spectroflow 773 variable wavelength UV detector	WISP 710B and Integrator
3	Beckman 110A	Kratos Spectroflow 773 variable wavelength UV detector	WISP 710B and Integrator
4	Perkin Elmer series 3	Perkin Elmer 65 T variable wavelength UV detector with temperature controlled oven	WISP 710B and Integrator
5	Beckman 110B	Perkin Elmer 650-S fluorescent detector	WISP 710B and Integrator
6	Beckman 110B	Perkin Elmer 204-S fluorescent detector	WISP 710B and Integrator
7	Beckman 110B	Hitachi 1000 fluorescent detector	WISP 710B and Integrator
8	Rainin HPLX	Shimadzu RF-535 fluorescent detector	
9	Rainin HPLX	Shimadzu RF-535 fluorescent detector	

samples of the drug in biological samples. Other regression methods, including weighted linear regression, have been investigated for use in selected assays. The parameters of the standard curve are used to convert the peak height ratio of the drug peak to the internal standard peak in a chromatogram to the drug concentration of the clinical specimen.

Six methodologies for drugs and metabolites were under development or reports were in preparation or revision during the fifth year of the contract. Analytical methodologies that were at various stages of completion during the past year in this laboratory are for the determination of the concentrations of the anti-viral drug, ribavirin, and WR 249,992 in plasma;the anti-malarial drug, ß-arteether, and dihydroginghaosu in biological fluids, the anti-malarial drug, halofantrine, and its metabolite, WR 178,460, in blood and plasma; WR 6026 and the mono desethyl metabolite of WR 6026 (WR 211,789) in biological fluids; the carboxyl metabolite of mefloquine, WR 160,972, in plasma; and the antimalarial drug artelinic acid in biological fluids.

Routine assays for nine studies were underway during the fifth year of the contract. Analysis of 470 human plasma and 468 human blood samples for the World Health Organization was reported in Analysis Report No. Hal/BP 89-7A, "Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Blood and Plasma Samples Obtained from the Protocol Titled 'Phase III Comparative Clinical

Trial of 4 Regimens of Halofantrine and Chloroquine in Treatment of *P. falciparum* Malaria.'" Analysis of 1258 human plasma and 16 infusate samples was reported in Analysis Report No. Pyr/P 89-8A, "Routine Analysis of Pyridostigmine Plasma Samples for the Protocol Titled 'Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Intravenous Pyridostigmine and Oral Doses of Standard and Sustained-Release Pyridostigmine in Healthy Men and the Influence of Food on Oral Pyridostigmine Pharmacokinetics.'" Analysis of 37 human plasma samples was reported in Analysis Report No. Pyr/P 90-2A, "Routine Analysis of Plasma Samples for Pyridostigmine (Free Base) Concentrations for the Protocol Titled "'Effect of Chronic Pyridostigmine Administration on Heavy Exercise in Hot Environments.'" Analysis of 18 human blood samples received from WRAIR was reported in Analysis Report No. Mef/B 90-3A, "Routine Analysis of Blood Samples for Mefloquine (Free Base) Concentrations." Analysis of 142 human plasma samples was reported in Analysis Report No. Pyr/P 90-4A, "Routine Analysis of Plasma Samples for Pyridostigmine (Free Base) Concentrations for the Protocol Titled 'Effects of Pyridostigmine Pretreatment on Physiological Responses to Heat and Moderate-to Intense Exercise'". Analysis of 434 beagle dog plasma, 429 beagle dog blood and 20 dosing solution samples was reported in Analysis Report No. Hal/BP90-5A, "Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Plasma and Blood Samples Obtained under the Protocol Titled 'Pharmacokinetics of Intravenous Halofantrine HCl.'" Analysis of 13 human plasma samples was reported in Analysis Report No. WR6/PB 90-6A, "Routine Analysis for WR 6026 and WR 211,789 (as Free Bases) of Plasma Samples Obtained from WRAIR - Preliminary Report." Analysis of 31 dog plasma samples received Jan. 30, 1991 will be reported for Study Hal/P 91-1A, "Analysis of Plasma Samples to Check Dilution Procedures." Analysis of 23 dog plasma and 48 dog blood samples received March 5, 1991 will be reported for Study Hal/BP 91-2A, "Analysis of Blood and Plasma to Verify *in vitro* Metabolism of Halofantrine and Partition of Halofantrine and WR 178,460."

DOCUMENTATION

Submittals Related to Contract DAMD17-86-C-6150 for 1990-91

Annual Reports

No. 4: covering 1989-1990, submitted May 15, 1990

Project Status Reports

No. 17, dated July 9, 1990
No. 18, dated Oct. 3, 1990
No. 19 dated Feb. 7, 1991
No. 20, dated April 2, 1991

Submittals Related to Contract DAMD17-86-C-6150 for 1990-91 (continued)

Study Reports

No. 15A: Ribavirin and WR 249,992 in plasma, dated Dec. 19, 90
No. 17A: Halofantrine and WR 178,460 in plasma and blood, dated Apr. 25, 90.

Analysis Reports

Hal/BP 89-7A: Halofantrine in plasma and blood, dated June 27, 1990.
Pyr/P 89-8A: Pyridostigmine in plasma, dated November 11, 1990.
Pyr/P 90-2A: Pyridostigmine in dog plasma, dated September 11, 1990.
Mef/B 90-3A: Mefloquine in blood, dated February 12, 1991.
Pyr/P 90-4A: Pyridostigmine in plasma, dated February 20, 1991.
Hal/PB 90-5A: Halofantrine in dog plasma and blood, dated March 20, 1991.

Publications

Planned papers

Ion-paired Liquid Chromatographic Method for the Analysis of Urine for Pyridostigmine.

Quantitation of WR6026 by HPLC.

STATUS OF ACCOMPLISHMENTS

The following review summarizes progress made on each of the methodologies under development and describes the routine analyses initiated during the fifth year of the contract.

RIBAVIRIN AND AV 206

Third and six month tests of stability for ribavirin and AVS 206 at -20°C were performed. Draft 1 of Study Report 15, "Quantitation of Ribavirin and WR 249,992 (free base) in Plasma by HPLC with C18 Bonded Silica Gel Columns and Acidic Aqueous Mobile Phases" was submitted on December 19, 1990.

DIHYDROQINGHAOSU AND ARTEETHER

Tests for development of assays for WR 255,131 (ß arteether) and dihydroqinghaosu involved electrochemical detection and the same mobile phase (with the addition of 0.3% H_3PO_4 and 20% CH_3CN at pH = 3) and the same phenyl column described in *J. of Chrom.* 414(1987)77-90. The use of precolumn photoirradiation and peroxide in the mobile phase have been abandoned on advice

from BAS that such a system produces a very high baseline. Following our literature search (*J. Chromatogr.* **493**, (1989) 125-136, very little else was found) for information of ß-arteether reactions, we tried 5 M HCl, which formed 8-methyl-5(2-propanalyl)decalin-4-ene-3-one. However, too many other peaks from this reaction were observed. A reaction mixture of H_3PO_4 (concentrated) and CH_3OH (v/v 1:2), which was expected to be somewhat less reactive but to give a similar breakdown product, appeared promising with 3-4 ng/0.1 ml of ß-arteether and half that of the dihydro compound observed by UV detection. A reaction mixture of H_3PO_4 (concentrated)/water (1:3) and of 10 N H_2SO_4 was examined. Extraction of ß arteether from buffered solutions by hexane worked better than dichloromethane. The internal standard, then, was antipyrine, with a retention time of about 7 min. Antipyrine didn't undergo treatment with the acid reaction mixture, since an acid treatment product had a retention time of 9 min and the retention time of ß arteether was 9.8 min. Plasma samples were being run and sample preparation setup was in the preliminary test stage.

Method development was halted at the COR's direction during the September 15 - December 14, 1990 reporting period. Also at the COR's direction, the planned Study Report 16 will not be prepared. Instead, findings for this project will be incorporated in Study Report 20, the artelinic acid assay methodology project (see below).

HALOFANTRINE

Improvements to the halofantrine assay methodology were submitted in draft 1 of Study Report No. 17, dated April 25, 1990, which contains validation results for halofantrine and WR 178,460 (as free bases) in blood and plasma. Analysis of additional blind blood and plasma samples supplied by WRAIR for measurement of accuracy of the halofantrine and WR 178,460 (as free bases) assay are underway.

Human blood (468) and plasma (470) samples were received October 5, 1989 in accordance with the protocol titled "Phase III Comparative Clinical Trial of 4 Regimens of Halofantrine and Chloroquine in Treatment of *P. falciparum* Malaria." Analytical results were reported in Analysis Report Hal/BP 89-7 (report submitted June 27, 1990).

Beagle dog blood (257) and plasma (258) samples were received April 18, 1990 in accordance with the protocol titled " Pharmacokinetics of Intravenous Halofantrine HCl." Final results (as Hal/BP 90-1 data) were faxed to WRAIR on February 4, 1991. Beagle dog blood (172) and plasma (176) samples were received September 19, 1990 in accordance with the protocol titled " Pharmacokinetics of Intravenous Halofantrine HCl." Final results (as Hal/BP 90-5 data) were faxed to WRAIR on December 18, 1990. Both sets of data (Hal/BP 90-1 and Hal/BP 90-5) were submitted on March 20, 1991 in draft 1 of Analysis Report Hal/BP 90-5.

Plasma (31) samples were received January 30, 1991. Preliminary results as study Hal/P 91-1 were faxed to WRAIR on February 14, 1991. A study on the effect of dilution of samples is being performed. Results will be combined with data from study Hal/P 91-2 and reported in Analysis Report Hal/P 91-2.

Plasma (22), blood (46) and red blood cell (22) samples spiked with halofantrine or WR 178,460 or obtained from a dog infused with halofantrine and blank blood (2), plasma (1) and rbc (1) samples were received March 5, 1991. Analysis of samples to determine metabolism and partition of halofantrine and WR 178,460 is currently underway. Results will be reported in Analysis Report Hal/P 91-2.

PYRIDOSTIGMINE

The laboratory in June 1990 received 1258 human plasma samples obtained according to the protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Intravenous Pyridostigmine and Oral Doses of Standard and Sustained-Release Pyridostigmine in Healthy Men and the Influence of Food on Oral Pyridostigmine Pharmacokinetics." Preliminary results were faxed to WRAIR on August 29, 1990. Draft 1 of Analysis Report Pyr/P 89-8 was submitted for approval on November 13, 1990. Infusate results were faxed to WRAIR on December 18, 1990.

The laboratory on May 15, 1990 received 37 human plasma samples in accordance with the protocol titled "Effects of chronic pyridostigmine administration on heavy exercise in hot environments." Final results were faxed to WRAIR on August 10, 1990 and were reported in draft 1 of Analysis Report Pyr/P 90-2 on September 11, 1990.

The laboratory on September 5, 1990 received 145 human plasma samples in accordance with the protocol titled "Effects of pyridostigmine pretreatment on physiological responses to heat and moderate-to-intense exercise." Preliminary results were faxed to WRAIR on September 19, 1990. Final results were faxed to WRAIR on February 1, 1991 and draft 1 of Analysis Report Pyr/P 90-4 was submitted to WRAIR on February 20, 1991.

WR 6026
AND WR 211,789 (WR 6026 METABOLITE)

Pentane, methyl *t*-butyl ether and methylene chloride were tested as extraction solvents to recover the metabolite from plasma. Method development for a plasma and blood method for determination of WR 6026 and WR 211,789 will be described in Study Report 18. Briefly, methyl *t*-butyl ether is used as extraction solvent to recover the metabolite from plasma and blood samples. Glassware must be silanized. An acetonitrile/water (60:40, v/v) mobile phase, a silica gel column, and UV detection at 263 nm are used. Standard curve calibrator concentrations for WR

6026 range from 0.980 to 98.0 ng/ml and for WR 211,789 range from 1.20 to 120 ng/ml.

Plasma (13) samples were received October 20, 1990 from WRAIR. An interference with the metabolite delayed immediate analysis of the samples. The interference peak is not observed when samples are extracted for a shorter length of time. Final results were faxed to WRAIR on February 13, 1991. Results will be presented in Analysis Report WR6/PB 90-6.

MEFLOQUINE & WR 160,972 (MEFLOQUINE METABOLITE) IN BLOOD AND PLASMA

C8 columns from Beckman and Whatman appear acceptable for separation of WR 160,972 (the mefloquine metabolite) from mefloquine. Use of methyl *t*-butyl ether to extract from plasma leaves interfering junk peaks. The next step will be to try several combinations of extraction solvents. The analyst has requested another sample of mefloquine (WR 142,490) for development of this assay. In addition, tests on the stability of mefloquine in blood are being performed. This project has relatively low priority. Stability, recovery and precision data were presented in Project Status Report 19, dated February 7, 1991. When completed, method development will be reported in Study Report 19.

The laboratory on July 11, 1990 received 18 blood samples for determination of the mefloquine free base concentrations. Corrected final results were faxed to WRAIR on February 4, 1991. The method used was modified from the plasma method described in Study Report No. 14, "Quantitation of Mefloquine (Free Base) in Plasma by High-Performance Liquid Chromatography, Extraction Method." The blood method primarily differs from the plasma method in sample preparation by:

1. Allowing blood standard curve calibrator samples to equilibrate for 1 hour following spiking with mefloquine working solutions;

2. Addition of 0.5 ml water; and

3. Sonication for 10 min prior to addition of internal standard.

ARTELINIC ACID

The mobile phase reported in Project Status Report 19 consisted of acetonitrile/water (50:50, v/v) and 0.5% phosphoric acid. A C18 column (250x4.6 mm, 5 µm particle size) was used. The flow rate was 1.5 ml/min. UV detection wavelength was 236 nm. Artelinic acid eluted at 20 min. C18 Bond Elut™s were washed with water, acetonitrile, methanol and water. Then, the sample (0.5 ml plasma) was loaded onto the Bond Elut™, washed with water, and eluted with methanol. The eluent is evaporated and redissolved in 300 µl of methanol. Internal standards investigated (with corresponding retention times in min when available) were: Qinghaosu (7.3), artesunic acid (6.5), arteether (38), and DQHS ß-

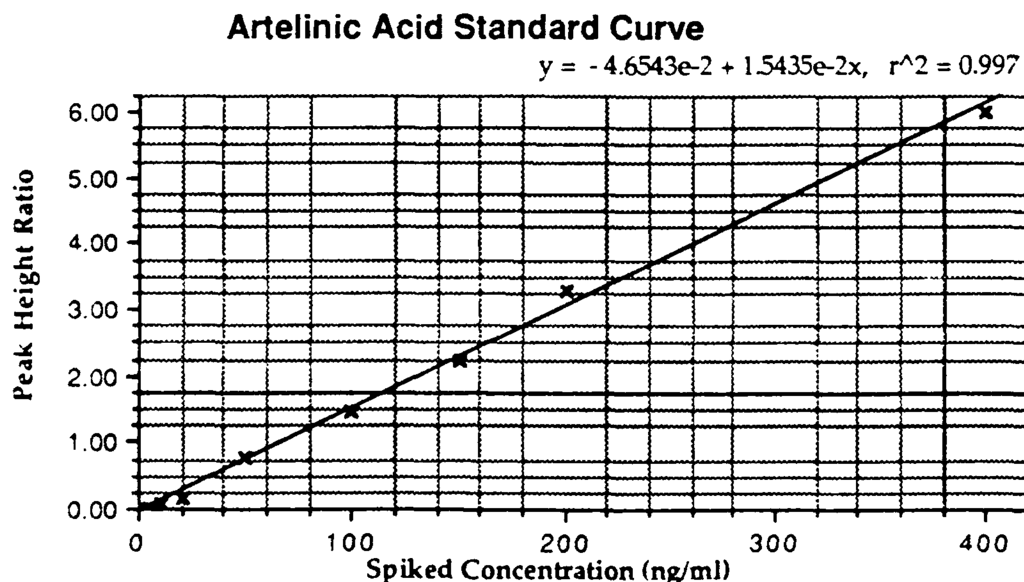
propyl ether (65). Ibuprofen, phenoprofen, ketoprofen and benoxyprofen were also tried, but all had retention times earlier than artelinic acid. If another compound with a usable retention time is not found, arteether can be used in combination with a gradient pump.

The mobile phase reported in Project Status Report 20 consisted of methanol/water (70:30, v/v) and 0.25% phosphoric acid. A C18 column (250x4.6 mm, 5 µm particle size) was used. The flow rate was 1.5 ml/min. UV detection wavelength was 238 nm. The sample (0.5 ml plasma) was extracted twice (vortex 5 min) with 2 ml methyl *t*-butyl ether. The sample's aqueous layer was frozen on dry ice/methanol. The organic phase was separated, then evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 µl methanol/water (50:50, v/v), and 70 µl was injected onto the column. Artelinic acid eluted at 29 min and arteether (the internal standard) at 31 min.

The latest standard curve (5 ng/ml will probably not be used in future curves)

Spiked Concentration (ng/ml)	Peak Height Ratio
0	0
5	0.0146
10	0.0655
20	0.1547
50	0.7705
100	1.4548
150	2.2309
200	3.2972
400	6.025

An artelinic acid standard curve.



TESTS

The following tests are conducted for the validation of a methodology under development. The sensitivity of the method is demonstrated by the analysis of prepared samples spiked at the drug concentration of the low point of the standard curve. Linearity of the method is demonstrated by obtaining acceptable spiked vs. calculated (or vs. peak response ratios), y-intercept , and coefficient of determination (r^2) values from the HPLC analysis of prepared standard curve samples. The accuracy of the method is demonstrated by the analysis of blind samples provided by the US government. The reproducibility of the method is demonstrated by interday and intraday analysis of prepared replicate samples spiked at several concentrations. The recovery of the method is determined by comparison of the analyzed concentration of the drug spiked in a reference solution versus the analyzed concentration of the drug spiked in biological specimens. The stability of the drug in specimens is determined by analysis of samples that have been frozen for various lengths of time.