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Analysis of Investigational Drugs in Biological Fluids - Method Development and Routine assay

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Using the procedures described in this report, we were able to work sequentially or simultaneously on eleven projects (1-WR 238,605, 2-halofantrine (and its metabolite), 3-WR 6026 (and its metabolites), 4-mefloquine (and its metabolite), 5-artelinic acid, 6-*p*-aminoheptanophenone (and related compounds), 7-primaquine (and its metabolite), 8-gentamicin and paromomycin, 9-pyridostigmine, 10-chloroquine (and its metabolites), and 11-a multiple drug interaction study in dog plasma for WR 238,605, mefloquine, chloroquine, quinine, doxycycline, and halofantrine with additional work on development and validation of LC/MS/MS methods for halofantrine (and its metabolite), WR 238,605) in terms of method development, validation, and characterization. We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA).

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INTRODUCTION

This report describes technical work accomplished and information gained in performance of contract number DAMD17-92-C-2028, titled "Analysis of Investigational Drugs in Biological Fluids - Method Development and Routine Assay," for the US Army Medical Research and Materiel Command (USAMRMC).

For many years our research group has been actively involved in the development of analytical methods to assay for drug substances in biological fluids for pharmacokinetic, bioavailability, drug metabolism and drug monitoring studies. This report describes the approach we took to develop sensitive (nanograms per milliliter of biological matrix), specific and quantitative analytical methods to support pharmacokinetic and bioavailability studies of candidate chemical warfare antidotes, antiparasitic drugs, radioprotectants and anti-infectious disease drugs.

In addition, routine analyses of biological specimens to support pharmacokinetic and bioavailability studies as part of preclinical and clinical investigations undertaken for the purpose of new drug development were performed as a significant adjunct to method development objectives. Within our routine analysis laboratory, we developed the capability to assay up to 10,000 samples per year.

There are many reasons for the U.S. military to develop various new drugs to protect or to treat soldiers confronted with the hazards of the modern battlefield. Like any pharmaceutical company, however, the military has to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA). Therefore, a great deal of work involving animal studies, preclinical and clinical trials, toxicity, metabolism and formulations must be carried out before a drug can be tried in the field. All of these studies depend on the adequacy of the analytical method for the particular compound. The route of administration and the dosage form are not necessarily the same in the field as in the clinic. For example, pyridostigmine is given prophylactically in the field, but the dose and route of administration are different for the treatment of myasthenia gravis or in anesthesiology.[1] Since military personnel are constantly involved in areas where they can be infected by parasites, including tropical or subtropical zones with drug-resistant forms, the U.S. Army needs to organize programs so that highly active and more effective new drugs can be discovered. These types of programs are generally ignored by private industry due to limited markets and profits.

This contract has offered us an interesting and stimulating challenge to utilize and extend our considerable capabilities to conduct method development and routine analysis in support of pharmacokinetic and

bioavailability studies. Our participation in this contract was possible by virtue of the experience and expertise of our staff in the area of pharmacokinetics, which requires assurance of extensive and rigorous internal and external analytical quality. As a result of our extensive involvement in these analytical programs, the staff members working on this project are the best in the field and have acquired a broad range of experience in the analysis of organic compounds in diverse media.

NATURE OF PROBLEM

Using the experimental procedures described in this report, we maintain the capability to complete projects on up to one new compound per year in terms of method development, validation, and characterization. We demonstrate sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describe the extent of recovery for the method, and report on the stability of compounds of interest in specimens during storage and drug analysis. Validation of sensitive and specific analytical methods follow procedures described in the Analytical Section Procedural Manual, Procedure 2D-3.1 and earlier versions "Procedure for Validation." Methods developed are such that a single technician can complete at least 15 clinical samples in one day. These methods are robust and portable enough to be transported to other laboratories. Within our routine analysis laboratory, we maintain the capability to assay up to 5,000 samples per year. Routine sample analysis will be performed in accordance with applicable procedures described in the Analytical Section Procedural Manual, Procedure 2D-4.2 and earlier versions. We have sufficient equipment and personnel to develop several candidate agents simultaneously and to be able to respond to changing priorities. We prepare and submit required reports in accordance with the contracted schedule.

BACKGROUND OF PREVIOUS WORK

Studies conducted over the 8 years prior to contract DAMD17-92-C-2028 under previous contracts including DAMD17-86-C-6150, DAMD17-85-D-0008, and DAMD17-83-C-3004 are listed in Tables 1 (study reports) and 2 (routine analyses reports).

TABLE 1: PREVIOUS STUDY REPORTS

Report No.	Report Date	Report Title	Test Article	Test System	Minimum Quantitation Limit
01	8/26/83	Analytical Procedure for the determination of WR 6026 in Plasma	WR 6026 WR211,789•2HCl WR 6026 WR211,789•2HCl	Plasma Plasma Blood Blood	6.44 ng/ml 8.00 ng/ml 6.44 ng/ml 8.00 ng/ml
03	1/22/85	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma	Pyridostigmine	Plasma	1.4 ng/ml
04	8/23/85	Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite, WR 178,460, in Blood and Plasma	halofantrine WR 178,460 halofantrine WR 178,460	Plasma Plasma Blood Blood	0.900 ng/ml 1.40 ng/ml 0.900 ng/ml 1.40 ng/ml
05	7/21/86	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma Using Silica Gel Column and an Aqueous Mobile Phase	Pyridostigmine	Plasma	1.39 ng/ml
06	1/8/88	High Pressure Liquid Chromatography (HPLC) of Mefloquine in Plasma	Mefloquine	Plasma	10.0 ng/ml
07	1/12/88	High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Urine	Pyridostigmine	Urine	13.7 ng/ml
08	9/23/88	High Pressure Liquid Chromatography (HPLC) of Physostigmine in Plasma with Ultraviolet Detection	Physostigmine	Plasma	1 ng/ml
09	9/12/88	Quantitation of Physostigmine & Eseroline in Plasma by HPLC with Fluorescence Detection	Physostigmine eseroline	Plasma Plasma	0.1 ng/ml 0.1 ng/ml
10	9/14/89	Quantitation of WR 6026 (Free Base) in Plasma & Blood by HPLC	WR 6026 WR 6026	Plasma Blood	0.980 ng/ml
11	9/28/89	Quantitation of WR 2721 in Plasma by HPLC with Electrochemical Detection	WR 2721	Plasma	0.100 µg/ml

TABLE 1: PREVIOUS STUDY REPORTS (Continued)

Report No.	Report Date	Report Title	Test Article	Test System	Minimum Quantitation Limit
12	11/14/89	Quantitation of WR 3689 in Plasma by HPLC with Electrochemical Detection	WR 3689	Plasma	0.0990 µg/ml
13	11/17/89	Quantitation of WR 238605 by HPLC	WR 238,605 WR 238,605	Plasma Blood	0.815 ng/ml 1.91 ng/ml
14	8/29/89	Quantitation of Mefloquine (f.b.) in Plasma by HPLC, Extract. Meth	Mefloquine	Plasma	8.00 ng/ml
15	12/19/90	Quantitation of Ribavirin and WR 249,992 (free base) in Plasma by HPLC with C18 Bonded Silica Gel Columns and Acidic Aqueous Mobile Phases	Ribavirin WR 249,992	Plasma Plasma	20 ng/ml 10 ng/ml
14	8/29/89	Quantitation of Mefloquine (Free Base) in Plasma by HPLC, Extraction Method	Mefloquine	Plasma	8.00 ng/ml
15	12/19/90	Quantitation of Ribavirin and WR 249,992 (f. b.) in Plasma by HPLC with C18 Bonded Silica Gel Columns and Acidic Aqueous Mobile Phases	Ribavirin WR 249,992	Plasma Plasma	20 ng/ml 10 ng/ml
16	Canceled	β-arteether project canceled	WR 255663		
17	4/25/90	Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	halofantrine WR 178,460 halofantrine WR 178,460	Plasma Plasma Blood Blood	0.960 ng/ml 0.928 ng/ml 0.960 ng/ml 0.928 ng/ml
18	Status report: 7/31/91	Quantitation of WR 6026 and WR 211,789 (WR 6026 Metabolite) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	WR 6026 WR 211789	Plasma Blood	0.980 ng/ml 1.21 ng/ml
19	Status report: 1/14/92	Tentative title: Quantitation of Mefloquine and its Metabolite, WR 160972 in Biological Fluids	mefloquine WR 160972	plasma blood	7.36 ng/ml -
20	Status: 10/16/ 91	Tentative title: Quantitation of Artelinic acid in Plasma	Artelinic acid	plasma	20.2 ng/ml

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.	
Routine Analysis of Halofantrine Plasma Samples Obtained from Protocol Titled "The Relative Bioavailability of Three Oral Formulations of Halofantrine Hydrochloride"	10/23/87	Halofantrine WR 178,460	plasma plasma	971 971	AY	86-1D
Phase III Comparative Clinical Trial of 4 Regimens of Halofantrine and Chloroquine in Treatment of P. falciparum Malaria	6/27/90	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood	470 470 468 468	Hal/BP	89-7
Routine Analysis for Protocol Titled "Pharmacokinetics of Intravenous Halofantrine HCl"	12/18/90	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood sol'ns	434 434 429 429 20	Hal/PB	90-5
Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Plasma Samples Obtained under the Protocol Titled "52-Week Chronic Oral Toxicity Study of WR 171,669 HCl (Halofantrine Hydrochloride) in Dogs"and "Analysis of Blood and Plasma to Verify in vitro Metabolism of Halofantrine and Partition of Halofantrine and WR 178,460"	7/16/91	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood	83 83 48 48	Hal/P	91-1&2
Routine Analysis of Plasma and Blood Samples for the Protocol Titled 'Disposition Kinetics of IV Desbutyl Halofantrine and the Effects of Gastric pH on the Bioavailability of Halofantrine-HCl'	2/4/92	Halofantrine WR 178,460 Halofantrine WR 178,460	plasma plasma blood blood dosing sol'ns	756 756 754 754 18	Hal/BP	91-3

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.	
Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Plasma Samples Obtained under the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR 171,669•HCl (Halofantrine Hydrochloride) in Rats"	9/23/91	Halofantrine WR 178,460	plasma plasma	118 118	Hal/P	91-4
Routine Analysis for Halofantrine and WR 178,460 (free bases) in Blood Samples Obtained for the Protocol Titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria"	1/21/92	Halofantrine WR 178,460	blood blood	107 107	Hal/B	91-5
Routine Analysis of Mefloquine Plasma Samples obtained from Six Clinical Protocols from Thailand	2/25/88	Mefloquine	plasma	781	Mef/P	87-1B
Routine Analysis of Plasma Samples from Thailand for Mefloquine Concentrations	12/7/88	Mefloquine	plasma	388	Mef/P	88-11
Routine Analysis of Blood Samples for Mefloquine (Free Base) Concentrations	2/12/91	Mefloquine	blood	18	Mef/B	90-3
Routine Analysis of Physostigmine Plasma Samples from the Protocol Titled "Bioavailability and Pharmacokinetic Study of Physostigmine (WR 006570) in Beagle Dogs"	8/26/88	Physostigmine Eseroline	plasma plasma	198 198	Phy/P	88-5
Routine Analysis of Physostigmine Plasma Samples from the Protocol Titled "Bioavailability and Pharmacokinetic Study of Physostigmine (WR 006570 AM) in Rhesus Macaques"	9/15/88	Physostigmine Eseroline	plasma plasma	196 196	Phy/P	88-6

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.	
Pilot Study - Analysis of Rat Plasma	9/14/88	Physostigmine Eseroline	plasma plasma	45 45	Phy/rP	88-8
Pilot Study - Analysis of Rat Perfusate	9/14/88	Physostigmine	perfus	37	Phy/rP r, pilot	88-9
Pilot Study - Analysis of Monkey Plasma	5/5/88	Physostigmine Eseroline	plasma plasma	8 8	"Phy/m P, pilot"	88-10
Routine Analysis of Physostigmine (free base) and Eseroline (free base) Rat Plasma Bile, and Tube Binding Samples for Samples Obtained from WRAIR	1/18/90	Physostigmine Eseroline Physostigmine Eseroline	plasma plasma bile etc bile etc	92 92 20 20	Phy/rP, pilot	89-6
Pyridostigmine in plasma (Israel)	5/14/86	Pyridostigmine	plasma	427	PY	85-4
"Pyridostigmine in plasma" (PY85-6-2 and PY85-6-3 combined) (Johns Hopkins, Millers)	7/3/86	Pyridostigmine	plasma	32	PY	85-6-4
Routine Analysis of Pyridostigmine Plasma Samples from Batelle Laboratories-MREF Protocol 27 (Battelle)	7/9/86	Pyridostigmine Pyridostigmine	plasma plasma, blind	648 22	PY	85-2-3
Routine Analysis of Pyridostigmine Plasma Samples Obtained from Protocol Titled "Pharmacokinetics of Orally Administered Pyridostigmine and Comparative Bioavailability of Liquid and Tablet Formulations" (Subjects 1-30)	12/3/86	Pyridostigmine Pyridostigmine	plasma dose sol	1698 12	PY	85-1
"Pyridostigmine in plasma (Johns Hopkins,Sub.1-24)"	1/12/87	Pyridostigmine pyridostigmine	plasma infusate	969 23	PY	85-6-5
"Pyridostigmine in plasma (Johns Hopkins,Sub.1-24)"	3/12/87	Pyridostigmine Pyridostigmine	plasma dose sol	1102 27	PY	85-6-6B

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.	
Routine Analysis of Pyridostigmine Plasma Samples obtained from Protocol Titled"Development of a Primate Model for Evaluating Efficacy of Treatment Regimens Against Nerve Agent Poisoning:Part I: Pharmacokinetics of Pralidoxime Chloride, Atropine Sulfate, and Pyridostigmine Bromide" (PY85-3-1 through PY85-3-5 combined)	5/29/87	Pyridostigmine	plasma, monkey	439	PY	85-3-6B
Battelle Rat Study Pyridostigmine in plasma (revised letter report)	7/28/87	Pyridostigmine	plasma, rat	102	none	none
Battelle Dosing Sol'ns Pyridostigmine in plasma (revised letter report)	7/28/87	Pyridostigmine	dose sol	92	none	none
Routine Analysis of Pyridostigmine Plasma Samples Obtained from Protocol Titled "14 day pilot dose range oral toxicity study in dogs" (Battelle)	7/30/87	Pyridostigmine Pyridostigmine	plasma dose sol	152 2	PY	85-2-2B
Pyridostigmine in plasma (Huntingdon dog)	9/30/87	Pyridostigmine	plasma dog	336	PY	85-5-3C
Routine Analysis of Pyridostigmine Plasma Samples obtained from Protocol titled "Comparative Bioavailability Studies of Pyridostigmine Bromide in Male Beagle Dogs " (31 July 1985) (Huntingdon dog)	10/7/87	Pyridostigmine	plasma, dog	324	PY	85-5C

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.	
Routine Analysis of Pyridostigmine Urine Samples from Protocol Titled "Bioavailability of Oral Pyridostigmine and Inhibition of Red Blood Cell Acetylcholinesterase by Oral and Intravenous Pyridostigmine"	2/3/88	Pyridostigmine	urine	110	Pyr/U (renamed from AY86-3)	86-3B
Routine Analysis of Pyridostigmine Plasma and Urine Samples from Protocol Titled "Pharmacokinetics and Pharmacodynamics of Sustained, Low-dose, Intravenous Infusions of Pyridostigmine"	2/24/88	Pyridostigmine Pyridostigmine Pyridostigmine	plasma urine infusate	498 72 24	Pyr/PU	87-2B
Routine Analysis of Pyridostigmine Plasma Samples from the Protocol titled "Comparative Oral Bioavailability Studies of Two Wax Matrix Formulations of Pyridostig- mine Bromide in Male Beagle Dogs"	3/29/88	Pyridostigmine	plasma	341	Pyr/P	88-1
Routine Analysis of Pyridostigmine Plasma Samples from the Protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of Sustained Release Pyridostigmine in Healthy Men," dated 9/18/87	8/3/88	Pyridostigmine	plasma	558	Pyr/P	88-2
"Routine Analysis of Pyridostigmine Plasma Samples from the Protocol titled ""Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of Sustained Release Pyridostigmine in Healthy Men,"" dated Sept. 30, 1987"	8/2/88	Pyridostigmine	plasma	476	Pyr/P	88-3

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.	
Routine Analysis for Protocol Titled "Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of Pyridostigmine Administered by an Osmotic-Delivery Module (osmetr) compared to Pyridostigmine Syrup in Healthy Men"	5/12/89	Pyridostigmine	plasma	374	Pyr/P	89-2
Routine Analysis for protocol titled "Safety, Tolerance, Pharmacokinetics and Pharmaco- dynamics of Single Oral Doses of a Commercial Formulation of Sustained-Release Pyridostigmine in Healthy Men."	5/16/89	Pyridostigmine	plasma	120	Pyr/P	89-3
Safety, Tolerance, Pharma-cokinetics and Pharmaco-dynamics of Intravenous Pyridostigmine and Oral Doses of Standard and Sustained-Release Pyridostigmine in Healthy Men & the Influence of Food on Oral Pyridostigmine Pharmacokinetics	11/13/90	Pyridostigmine	plasma	1250	Pyr/P	89-8
Routine Analysis for Protocol Titled "Effect of chronic pyridostigmine administration on heavy exercise in hot environments"	9/11/90	Pyridostigmine	plasma	37	Pyr/P	90-2
Routine Analysis for Protocol Titled "Effects of Pyridostig-mine Pretreatment on Physio-logical Responses to Heat & Moderate-to Intense Exercise"	2/20/91	Pyridostigmine	plasma	142	Pyr/P	90-4
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharm-acokinetics and Methhemo-globin Pharmacodynamics in the Beagle Dog"	4/13/89	WR 238605 WR 238605	plasma blood	62 62	WR5/B P, pilot	89-1

TABLE 2: PREVIOUS ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Date	Test Article	Test System	No. of Samples	Report No.	
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	6/1/89	WR 238605 WR 238605	plasma blood	88 88	WR5/B P, pilot	89-4
Routine Analysis for protocol titled "Simultaneous Modeling of WR238605 Succinate Pharmacokinetics and Methhemoglobin Pharmacodynamics in the Beagle Dog"	8/25/89	WR 238605 WR 238605	plasma blood	240 240	WR5/B P	89-5
Routine Analysis of WR 6026 Plasma Samples Obtained from Clinical Protocol Titled "Single-Dose Absorption and Pharma- cokinetics of WR 6026 Hydrochloride in Healthy Subjects"	6/24/87	WR 6026	plasma	192	AY	86-2D
Routine Analysis of Blood Samples from the Protocol Titled "Multiple-Dose Pharmacokinetics, Safety and Tolerance of WR 6026 Hydrochloride in Healthy Subjects"	4/21/89	WR 6026	blood	571	Wr6/B	88-7
Routine Analysis for WR 6026 and WR 211,789 (as Free Bases) of Plasma Samples Obtained from WRAIR - Preliminary Report	2/13/91	WR 6026 WR 211789	plasma plasma	13 13	Wr6/PB	90-6

PURPOSE OF THE PRESENT WORK

Work on development and/or validation of analytical methodologies during the current contract focused on assays for WR 238,605 (and its stereoisomers), halofantrine (and its metabolite and their stereoisomers), WR 6026 (and its metabolites), mefloquine (and its metabolite), artelinic acid, *p*-aminoheptanophenone (and related compounds), primaquine (and its

metabolite), gentamicin and paromomycin, pyridostigmine, WR 242511, chloroquine (and its metabolites), WR 243,251, quinine and doxycycline. Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of WR 238,605 (and its stereoisomers), halofantrine (and its metabolite and their stereoisomers), WR 6026 (and its metabolites), mefloquine (and its metabolite), *p*-aminoheptanophenone (and related compounds), primaquine (and its metabolite), gentamicin and paromomycin, pyridostigmine, chloroquine (and its metabolites), quinine, and doxycycline.

METHODS OF APPROACH

The general development plan is described with emphasis on the laboratory procedures used. Using the procedures described in this report, we were able to work sequentially or simultaneously on eleven projects (1-WR 238,605, 2-halofantrine (and its metabolite), 3-WR 6026 (and its metabolites), 4-mefloquine (and its metabolite), 5-artelinic acid, 6-*p*-aminoheptanophenone (and related compounds), 7-primaquine (and its metabolite), 8-gentamicin and paromomycin, 9-pyridostigmine, 10-chloroquine (and its metabolites), and 11-a multiple drug interaction study in dog plasma for WR 238,605, mefloquine, chloroquine, quinine, doxycycline, and halofantrine with additional work on development and validation of LC/MS/MS methods for halofantrine (and its metabolite), WR 238,605) in terms of method development, validation, and characterization. We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis. Validation of sensitive and specific analytical methods follow procedures described in the Analytical Section Procedural Manual, Procedure 2D-3.2 and earlier versions "Procedure for Validation of an Assay Methodology." Methods are developed such that a single technician could complete at least 15 clinical samples in one day. These methods are to be robust and portable enough to be transported to other laboratories.

All drug standards received from the USAMRDC were logged into our record book and stored as required (protected against light, heat, or moisture). If necessary, they were checked for chemical purity or radiopurity by high pressure liquid chromatography or thin layer chromatography, purified through recrystallization or chromatography, and hydroscopic samples were dried according to USP methods.

Sample Preparation for Assay Development

Spiked samples of biological media are prepared by spiking different amounts of drug from known stock solutions into the biological media. Samples are mixed, then equilibrated for up to one hour at room

temperature, unless the compound of interest is unstable, in procedures in which it is especially important for measuring drug concentrations in blood, since drugs may take some time to reach equilibrium with erythrocytes.

Sample Preparation Procedures

Suitable preparation of the biological specimens is essential for the successful application of an analytical technique. The preparation procedure should be as simple as possible, yet allow for the specific measurement of the drug in the presence of numerous biological components. The extent of sample work-up is therefore largely determined by the selectivity and sensitivity of the analytical technique. Interfering endogenous substances must be removed before analysis. A second objective in devising preparation steps for a biological specimen is to protect the analytical apparatus from contamination by proteins and undissolved particles. Biological sample preparation thus varies according to the technical demands of the various analytical instruments utilized. Since the advent of highly selective analytical methods that combine chromatographic separation and detection in one unit [e.g. HPLC], the importance of the second objective has become more critical.

Protein Precipitation

Protein precipitation methods are rapid; they involve mixing the sample with water-miscible organic solvents. Acetonitrile yields a protein precipitate that can be readily centrifuged into a small pellet. Use of protein precipitation alone, without further work-up, is a popular application in HPLC analysis. It is possible, using appropriate measurement devices such as electrochemical or fluorescence detectors, to obtain adequate sensitivity so that measurements in the nanogram per milliliter range can be made for drugs using small aliquots of the biological sample. We have used the protein precipitation method of sample preparation extensively in the development of analytical assays, including for antibiotics that are zwitterionic in nature, generally possess very low water-to-oil partition coefficients and, thus, are extremely difficult to extract efficiently. Also, protein precipitation is one method of choice for sample preparation, since a simpler sample preparation procedure reduces the risk of degradation. We use the direct protein precipitation method for our studies whenever possible (as demonstrated in Study Report 6 for mefloquine, Study Report 11 for WR 2721 and Study Report 12 for WR 3689).

Lower limits of quantitation with ultraviolet (UV) detectors are usually at about 50 ng/ml concentrations when the protein precipitation method is used. If UV detection is required, organic solvent extraction and solid phase extraction are more useful methods for preparation of biological samples for subsequent analysis. Extraction also limits column overloading and removes assay interferences.

Solvent Extraction

Three major variables were considered in the design of suitable organic solvent extraction procedures: the polarity of the organic solvent, the pH of the aqueous phase, and the volumes of the organic and aqueous phases (as demonstrated in Study Reports 8 and 9 for physostigmine and its metabolite eseroline in plasma and Study Report 10 for WR 6026). A higher pH is often desirable since many endogenous substances are acidic and will not be extracted at alkaline pH. Consideration of pH is therefore important even when assays are developed for neutral drugs. Lipophilic bases are quite uncommon in body fluids, so it should be relatively easy to analyze many of the lipophilic basic drugs by extracting at high pH (as shown in Study Report 13 for WR 238,605 in plasma and blood and Study Report 14 for mefloquine). However, one solvent partitioning step alone is not always capable of separating bases from acids and neutral compounds. In such cases, multiple extraction steps must be employed.

A sample preparation method combining protein precipitation with acetonitrile and extraction with organic solvent is also a viable option. This method has been successfully used in our halofantrine assay ([2] and Study Report 17).

Commercial prepacked solid phase columns [e.g. Bond Elut™] with different types of packing materials, such as silica, C2, C8, C18 and ion exchange were employed. These columns are very useful for sample purification. Two approaches can be utilized: 1] the column separates desired compound(s) from interferences, or 2] the column retains desired compound(s), undesired endogenous substances are washed away, and the desired compound(s) are eluted with a suitable solvent. For low nanogram or picogram per milliliter concentrations, the method of retaining the desired compound on the column is preferred. This method has been successfully used in our laboratory for charged, water soluble compounds (pyridostigmine (see Study Report 5 for plasma and Study Report 7 for urine)), or highly nonpolar lipophilic, weakly basic and nonvolatile compounds (WR 6026[3] and halofantrine[2]) in biological fluids. For example WR 6026 and halofantrine are non-polar lipophilic compounds which are retained on C8 columns. Pyridostigmine, a quaternary amine, will not elute with CH_3CN alone. A 2 ml CH_3CN wash after loading the biological sample onto the C8 column eliminates undesired substances. The drug is subsequently eluted with CH_3CN containing SDS and tetramethylammonium chloride (TMA+Cl-) or 1% HCl culminating in a quantitation limit of 2 ng/ml with UV detection.

Specific functional groups in molecules of interest can also be advantageously used to purify biological samples by solid phase extraction. Diol functional groups can adsorb on a boronate column and subsequently be eluted with an acidic solution. This turned out to be our method of choice in

the ribavirin and WR 249,992 assay development project (see Study Report 15).

Adsorption losses to glass or other apparatus for the low level lipophilic antimalarial drugs probably explains the inconsistent results reported by many investigators. The significance of this adsorption should be considered, especially when several extraction steps are to be employed. This was demonstrated during our development of the assay for halofantrine (WR 171,669) and its active metabolite, WR 178,460, in which WR 194,965 was used as the internal standard ([2] and Study Report 17). The compounds were adsorbed by the glassware after reconstitution of the extract with organic solvent. In our experience, a true measurement of drug was obtained with the addition of a small amount of surface active agent to the solvent system before delivery onto the HPLC column. Adsorption loss can also occur in the port of delivery.

Detector Selection

The detector is a device that supplies an output in response to the presence of the compound(s) of interest. It is connected to the outlet of the column to monitor the column effluent in real time. The detector can be the most sophisticated and one of the most expensive components of a chromatographic system. Optical detectors, which currently dominate the field for biological samples in HPLC, include UV-visible absorbance detectors and fluorescence detectors. Depending on the measured difference between incidental and transmitted light intensity, these instruments can detect down to 9 to 10 ng of sample if the direct precipitation method is used. Electrochemical (EC) detectors are also used for routine work due to their specificity and/or sensitivity.

UV-Visible Absorbance Detector

Since the analytical methods for this contract required the quantitation of nanogram per milliliter concentrations of drug in biological samples, samples assayed with the UV detector required an extensive extraction work-up. For example, the pyridostigmine plasma assay was capable of quantitating 2 ng/ml concentrations of pyridostigmine (free base) (see Study Report 5) with UV detection only because of the extensive extraction procedure.

Fluorescence Detector

Fluorescence detection is more selective than UV spectroscopy. However, more structural requirements must be met to produce a high fluorescence yield (Ø) and to allow measurement above a negligible background (i.e., better quantitation limits). Minimum detection limits for the fluorescence detector can extend below the nanogram per milliliter level for favorable samples. (See Study Reports 9, 13, and 17).

Fluorescence intensity can be manipulated both by changes in solvent components and the pH of the solvent system. For example, quinoline is non-fluorescent in hexane but fluoresces in ethanol, while indomethacin shows fluorescence at a pH above 12. Most of the synthetic antimalarial drugs are asymmetrically conjugated, not strongly ionic and, hence, would be expected to fluoresce. Fluorescence detection might therefore be expected to be the method of choice for measuring antimalarial drugs due to the sensitivity, selectivity and lower dependence on instrumental stability (from pressure and temperature changes) of the detector.

Two different light sources at various wavelengths are used in commercial fluorescence detectors. They are the deuterium and the xenon arc lamps. The xenon arc lamp has high intensity and the energy is more evenly distributed at different wavelengths, whereas the deuterium lamp emits at lower energy than the xenon arc and the intensity is drastically diminished at wavelengths above 280 nm.

Since the intensity of emitted fluorescence is dependent upon the intensity of the excitation source, it would appear that the sensitivity of a fluorescence assay can be increased without limit by using the most intense source. Many researchers do not realize that marked differences can be found with different lamp sources in commercial detectors.

8-Amino-quinoline antimalarial drugs, such as WR 6026, WR 238,605 and mefloquine (Study Reports 6, 10, 13, 14, 18, and 19) are highly conjugated and the excitation wavelengths were expected to be high. The xenon arc source equipped with monochronometers to collect both the excitation and emitted energy wavelengths provided us with maximum flexibility in fluorescence detection. With these devices, specific wavelengths for optimum sensitivity and/or selectivity were conveniently selected.

Electrochemical Detector

Electrochemical detectors (EC) are also used in methods of choice for applying liquid chromatography to trace (sub-nanogram) analysis. EC detection can provide the sensitivity and selectivity necessary for practical analytical procedures in a variety of situations. Material eluted from the chromatographic column acts at an electrode surface under controlled potential conditions and the current which results from the net exchange of electrons is monitored as a function of time. Since the amount of material converted by the electrochemical reaction is proportional to the instantaneous concentration, the current will be directly related to the amount of compound eluted as a function of time. The flow through a thin layer electrochemical cell is ideally suited for LC analysis since it can be easily constructed with a very small dead volume (1 µl) and maintain extreme sensitivity toward electroactive compounds. Several configurations using glassy carbon, carbon paste, or mercury-gold electrodes have been developed.

If chromatographic conditions are carefully controlled, EC detection is quite precise and quantitative data can be obtained at the picomole level (total injected amount) for many compounds. In addition to being extremely sensitive, the electrochemical detector is quite specific in that only compounds electroactive at a given potential are detected. A large number of extremely important endogenous compounds, drugs, drug metabolites, food additives and organic pollutants are electroactive and therefore can be studied by EC. It is the method of choice for the detection of catecholamines and their analogs; numerous assay methods using EC detection have been published in the recent literature. We have been successful in using this detector for measuring the morphine analog, nalbuphine in urine. When determining whether or not a particular compound can be successfully analyzed by EC, it is not sufficient to know that the compound can react electrochemically. The type of electrode surface, the nature of the solvent system and relative ease of oxidation or reduction must be carefully considered before one can ascertain whether such an analysis is feasible (see Study Reports 11 and 12 for phosphorothioate assays). Many important compounds have been studied in detail and conditions for analysis have been optimized. In order to assess fully the possibility of developing a new assay, it is desirable to carry out voltametric studies. This is equivalent to measuring an adsorption spectrum prior to using a UV detector.

With detection in the reductive mode, analysis of blood for artesunic acid and dihydroquinghaosu had been successfully carried out in Walter Reed Army Institute of Research.[4]

Phosphorothioates ($R\text{-}SPO_3H_2$) are potential radioprotective drugs investigated by the US Army. Neither UV nor fluorescence detection is suitable for this type of compound unless some other functional group in these molecules can be derivatized. To make matters worse, phosphorothioates are readily hydrolyzed to free sulfhydryl compounds in vivo (metabolism) and in vitro (degradation) and possibly further oxidized to disulfides. However, phosphorothioates can be detected by EC with dual mercury/gold electrode detectors connected in series. These can be very useful for the simultaneous determination of thiols and disulfides. Two Hg/Au electrodes are utilized in a series arrangement with reduction of disulfide to thiol at the upstream electrode, followed by conventional thiol detection downstream. The upstream electrode behaves as a novel on-line post column reactor of negligible dead volume. Phosphorothioates, thiols and disulfides are all readily quantitated in this detector and suitable separation is achieved by the HPLC system. It is interesting to recall that disulfide is actually being detected as the corresponding free thiol. No confusion occurs in measurements, however, because thiols are chromatographically resolved from the disulfide and thus separately detected.

LC/MS/MS

Mass spectrometric detectors are increasingly used in methods of choice for applying liquid chromatography to trace (sub-nanogram) analysis. Our Liquid Chromatographic/Mass Spectrometric/Mass Spectrometric (LC/MS/MS) system for analysis of biological specimens requires development and validation of procedures with a PE Sciex-API III® system that uses a short liquid chromatography column (5 µm particle size, 4.6 X 50 mm), the usual liquid chromatographic mobile phases, and mass spectrometric detection with sample inlet by heated nebulizer, positive ionization by APCI (Atmospheric Pressure Chemical Ionization) and mass scanning by MRM (Multiple Reaction Monitoring) analysis.

Solvent System and Column

One of the most important steps in the development of an HPLC assay is selection of a suitable solvent system (mobile phase) and stationary phase. They are both closely related for maximum separation. Practical approaches are discussed in this section.

Reverse-Phase and Bonded Phase Columns

We intended to use reverse-phase systems for the majority of the analytical methods developed for HPLC assay described in this contract, since such bonded phase columns have several advantages for applications involving biological fluids. Reverse phase columns are stable since the stationary phase is chemically bonded to the support and cannot easily be removed or lost during use. Therefore, a pre-column and/or pre-saturation of the two phases is/are not required. Reverse-phase columns have minimal irreversible retention which is compatible with a large variety of solvents; it is often possible to inject an aqueous sample without further treatment. As a result, bonded phase columns (BPC) are especially suited for samples containing components with widely varying K' (column capacity factor). The availability of a wide variety of functional groups on BPC packing allows reverse phase and ion paired chromatography to be carried out in a relatively simple, straight-forward manner.

In reverse-phase liquid chromatography, water is the polar solvent and any less polar, water-miscible solvent can be used in conjunction. Common examples of the second solvent include methanol, acetonitrile and tetra-hydrofuran. The design of a successful LC separation depends on matching the right mobile phase to a given column and sample ion pairing mode.

Aqueous Mobile Phase and Silica Columns

The recent use of an unbonded silica stationary phase and an aqueous mobile phase has been successfully used in our laboratory for the liquid

chromatographic separation of lipophilic amines. When C18 bonded phase columns are used, it is often necessary to employ amine mobile phase modifiers to ensure good retention times and peak shapes in the ion-suppression mode. Recent work suggests that unbonded silica gel, with the maximum concentration of surface silanol groups, is a preferable stationary phase for these compounds. Use of unbonded silica as the stationary phase permits the separation of a wide variety of amine compounds with a simple mobile phase containing an organic solvent and an aqueous phosphate buffer at neutral to alkaline pH. The retention volumes are lower and the peaks are more symmetrical when silica, rather than a C18 bonded support, is used as the stationary phase. The method is especially suitable for assays of biological fluids, since endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column while cationic aliphatic amine drugs will be retained. The interfering substances in biological fluids are eluted at the solvent front, leaving a very clean base line about the drug's retention time. Using a silica column and an aqueous solvent system, we obtained a quantitation limit of 2 ng/ml for plasma samples for pyridostigmine (free base) (see Study Report 05).

Selectivity and Resolution Modification

As a general approach to increasing (column selectivity) and improving resolution, several options are available and can be ranked in order of decreasing promise.

Modification of Mobile Phase

Many different properties of the solvent must be considered, including solvent strength and selectivity. Polar compounds are best separated by a polar solvent system, while non-polar compounds should be separated with a less polar system. Separation may be defined as the ability of the solvent system and column material to retain the compound of interest on the column for a longer period of time than the undesired components. We found that a change from methanol to acetonitrile can sometimes enhance the selectivity of the column. We tend to use acetonitrile as the solvent modifier since it has a lower viscosity and tends to increase the efficiency of the column; it is also characterized by increased miscibility with non-polar samples.

Change of pH and Ionic Strength

Aqueous buffers are commonly employed to suppress ionization of the ionizable sample components in reverse-phase analyses. The pH of the mobile phase is varied and the resulting changes in K' (column capacity) and alpha (column selectivity) are examined.

TABLE 3: DRUGS IN PLASMA ASSAYED WITH A SILICA GEL COLUMN AND AN AQUEOUS MOBILE PHASE'

Drug	Detection Limit (ng/ml)	Detection Mode	Sample Preparation	Mobile Phase	Retention Time (min.)
WR 238605	0.8	Fluorescence	Liquid extraction	50% CH_3CN 5 mM $(NH_4)_2HPO_4$ pH = 7.0	6.50
WR 6026	1.0	UV	Liquid extraction	60% CH_3CN 5 mM $(NH_4)_2HPO_4$ pH = 7.0	7.00
Halofantrine	1.0	Fluorescence	Liquid extraction	80% CH_3OH 5 mM $(NH_4)_2HPO_4$ pH = 8.2	7.00
Pyrido-stigmine	1.4	UV	Solid Phase extraction	50% CH_3CN, 0.05% TMAC 5 mM $(NH_4)_2HPO_4$ pH = 7.2	16.4
Mefloquine	8.0	UV	Liquid extraction	80% CH_3OH 5 mM $(NH_4)_2HPO_4$ pH = 7.5	10.1

Strength of buffers or ion-pairing agents can also influence the retention times of many drugs. Most of the antimalarial drugs are highly hydrophobic in nature, hence a high ratio of organic solvent modifier should be required and ion-pair techniques will be involved.

For silica stationary and aqueous mobile phase systems, the interaction between silica and amine is electrostatic and the separation mechanism is similar to an ion-exchange mechanism. Here, the pH of the mobile phase (pH 7-9.5) and pKa of the amine are very important in determining retention time, while the pH of the mobile phase in bonded phase systems (pH 2-5) is not as critical. Ionic strength is also critical. Thus for silica gel-aqueous mobile phase systems, mobile phase pH and ionic strength are more important to retention time determination than the organic modifier (e.g. CH_3CN, CH_3OH), which is the critical determinant in bonded gel - reverse-phase systems.

Change of Stationary Phase

A change of stationary phase is less convenient than a change in mobile phase composition and is less commonly used. Further adjustment of the

mobile phase composition is usually required when a new column packing or stationary phase is used to optimize solvent strength and K' values.

Most aromatic antimalarial drugs are very non-polar. It is reasonable to expect that the retention time will be shorter on a more polar C8 column (tend not to retain) than a non-polar C18 column. This was found to be true during the development of our first WR 171,669 (halofantrine) assay. The retention time for this compound is reduced by 1/3 by changing from a C18 column to a C8 column when the same mobile phase is used.

Temperature Change

The fourth technique for varying K' values is to increase (or decrease) the temperature. Since an increase in temperature normally reduces all sample K' values, it is usually necessary to decrease solvent strength to compensate for this effect. A change in temperature usually has little effect on sample K' values in liquid-liquid chromatography, but it is important in ion exchange and ion-pair chromatography. For this reason, a change in temperature for improvement of K' in ion-exchange and ion-pair chromatography is generally more promising than a change in stationary phase.

Complexation

A final means of changing K' values, sometimes dramatically, is through chemical complexation. A well known example is the use of metal ions (e.g. $Ag^+NO_3^-$) in the solvent system to separate various olefinic compounds. The complexation of olefin and metal ion causes dramatic changes in retention time and selectivity.[5] This technique is probably applicable for some antimalarial drugs.

For the most part, we intended to use C8 columns and/or ion-pair techniques to develop assay methodologies. However, silica gel column - aqueous mobile phase systems are our general method of choice for amines. Since measurement concentrations of 5 to 20 ng/ml are required, we expected to use 5 μm particle size columns for separation of drugs.

Derivatization

Derivatization is an important adjunct to HPLC assays. The choice of derivatization procedure is dependent upon the type of detector that is used. We are actively involved in pre-column and post-column derivatization as well as in structure modification studies to increase detection sensitivity. A wealth of information on potentially useful derivatives is available from the disciplines of qualitative organic analysis[6] and protective group synthesis.[7] In choosing a derivative for HPLC, ideally the reaction should be specific, quantitative, free from side reactions, complete in a relatively short time, and done under mild conditions. This kind of information is not readily available

in the literature, and therefore, derivatization studies can be a rather time consuming venture. The design or choice of a derivatizing agent is critical.

Post column derivatization or degradation is also an excellent way to increase sensitivity of an assay. The technique of post column hydrolysis at alkaline pH and post column oxidation reactions with potassium permanganate or potassium periodate can be applied to assays that employ fluorescence detection. Post-column photo-irradiation is another way to increase sensitivity. First, the drug of interest is separated from other components of the sample by HPLC. Then, the sensitivity is enhanced by photo-irradiation which may rearrange the chromophore or otherwise break bonds to form a fluorescent species

Assay Validation

Validation of the methods were performed using biological fluids obtained from same species, when possible. This process indicates sample stability, method precision, accuracy and selectivity, and the feasible sample concentration range for use in pharmacokinetic or bioavailability studies. Validation procedures are part of our standard operating procedures (SOP) which are written in accordance with our program to meet Good Laboratory Practice (GLP) regulations. The procedures are described in the Analytical Section Procedural Manual, Procedure 2D-3.2 "Procedure for Validation of an Assay Methodology." This section summarizes Procedure 2D-3.2.

Specificity

The specificity should be evidenced by showing with chromatograms that: Test compounds are separated from major metabolites (if metabolite standard is available); Test compounds are separated from co-administered drugs (if any); At least three different sources of biological fluid should be free of possible interference of endogenous peaks at the retention times of test compounds.

All assay methods developed required use of an internal standard. Analogs of the compounds under study or chemicals with similar functional groups were preferred as internal standards. The internal standard must elute at a different time than the drug of interest, yet separate from endogenous substances in the biological sample. In addition, it should have similar extraction or partition properties as the drug of interest during the sample preparation process.

Linearity

Linearity is demonstrated by acceptable spiked vs. calculated (or vs. peak response ratios), y-intercept, and coefficient of determination (r^2) values for the standard curve (concentration vs. response).

Calibration curves were constructed by using the peak height (or peak area) ratio of drug to internal standard versus spiked concentration of drug. The calibration curve was used for quantitation of the chemical sample concentration. Linear regression (unweighted or weighted method) to obtain calculated concentrations, correlation coefficient (r or r^2), slope of regression line, and y -intercept were used to evaluate sample concentrations.

In the weighted least squares linear regression method, weights (w) = $1/y_i$, the intercept, *b*, is defined by:

$$b = \frac{\left(\left(\sum w_i x_i^2\right)\left(\sum w_i y_i\right) - \left(\sum w_i x_i\right)\left(\sum w_i x_i y_i\right)\right)}{\left(\left(\sum w_i\right)\left(\sum w_i x_i^2\right) - \left(\sum w_i x_i\right)^2\right)}$$

and the slope, *m*, is defined by:

$$m = \frac{\left(\left(\sum w_i\right)\left(\sum w_i x_i y_i\right) - \left(\sum w_i x_i\right)\left(\sum w_i y_i\right)\right)}{\left(\left(\sum w_i\right)\left(\sum w_i x_i^2\right) - \left(\sum w_i x_i\right)^2\right)}$$

Two standard curves may be calculated from the same set of standard curve calibrators (unless the weighted linear regression method is used). The low range curve is calculated from low concentration standard curve points and is used to derive concentrations from samples with peak response ratios at or below the calculated peak response ratio of the highest standard curve point used in the low range curve. The high range curve is calculated from all standard curve points and is used to derive concentrations from samples with peak response ratios above the calculated peak response ratio of the highest standard curve point used in the low range curve.

The results are reported as a table containing spiked concentrations, peak response ratios, calculated concentrations, slope(s), intercept(s) and r^2 value(s) of a typical standard curve that was used in the method validation and as a table containing all slopes, intercepts and r^2 values of standard curves run in "intraday" and "interday" studies.

Lower Limit of Quantitation

The lower limit of quantitation is defined as the lowest standard curve concentration which can be reasonably, accurately, and precisely quantitated. Six samples spiked to the lowest standard curve concentration and a standard curve are prepared. The samples are run together within one day (or one run). The 6 lowest point of the standard curve sample concentrations, and their mean, S.D., C.V. (percent) and deviation (percent) are calculated. These data are used as the quantitation limit intraday result.

The 6 calculated lowest point of the standard curve concentrations that were obtained in the interday precision study and their means, S.D.s, C.V. percents and deviation percents are used as the quantitation limit interday result.

Recovery

It is important to check the recovery of compounds of interest during the assay in order to assess the uniformity of recovery during the assay or whether or not a better recovery can be obtained. Radio-labeled drugs, when necessary, were added to the sample and either the direct precipitation, solid phase purification or the extraction procedure was utilized to evaluate recovery. If labeled compounds were not available, a recovery study similar to those for WR 6026,[3] halofantrine and its metabolite, WR 178,460,[2] and pyridostigmine[8] were carried out. In brief, the recoveries of these drugs from plasma or whole blood were determined by comparison of the drug-to-internal standard peak height ratios of blood or plasma versus water samples spiked with the drug. In each case, the internal standard was added after sample was eluted from the solid phase column, extraction from organic solvent, or direct precipitation with CH_3CN to insure that the internal standard did not bind to the blood or plasma or to the cartridge during the preparation.

Precision

Precision is expressed as the standard deviation (S) of the assayed concentration where Xi are the repeated concentration measurements of an individual sample and $\overline{x}$ is the mean concentration.

$$S = \left(\frac{\sum_{i=1}^{N} (Xi - \overline{x})2}{(N - 1)} \right)^{\frac{1}{2}}$$

The coefficient of variation (C.V.) was used for determination of the precision. The sample number was 6 for intraday and 12 for interday precision. The bias of an assay method is determined by comparing biological sample results with spiked values. The significance of the bias is established by setting a confidence limit.

$$\text{Percent C.V.} = \frac{S \bullet 100}{\overline{x}}, \quad (N \geq 6)$$

If needed, the assay results were compared to those obtained with an assay of proven reliability and specificity. For example, the Pearson correlation coefficient (r) can be used. Maximum r value indicates exact correlation between the two variables and r = 0 indicates complete independence.

$$r = \frac{(Si - \overline{s})(Yi - \overline{y})}{n \bullet Sx \bullet Sy}$$

The within-run precision was determined by measuring the amount of drug in a number of biological samples, in duplicate. The duplicate mean results are used to calculate the standard deviation. The between-run precision is measured on separate days with replicate samples at low, intermediate and high drug concentrations. From these three sets of replicate samples, the between-run standard deviation is calculated for each drug concentration.

Accuracy

Accuracy was determined by assaying a series of blind samples prepared according to the project director of DAMD. Estimates of the accuracy of the method over the standard curve working range were also determined in the precision analysis by the analysis of replicate spiked samples for intraday (n = 6) and interday (n = 12) precision. Results were expressed as relative error (RE) with respect to the spiked concentration.

Stability in Biological Specimens

Stability studies of a drug in biological media serve to establish the procedure for proper storage of the samples and furnishes information to clinical researchers on how best to handle these occasionally labile samples. We have a great deal of experience in planning and executing the required stability studies. In methods developed for analytical and clinical studies, drug stability may play a particularly important role.

Known amounts of sample in different biological media are measured at various times after preparation and assayed for the drug, in duplicate. Variables, including light exposure, storage conditions (container type) and pH of the biological samples, are evaluated if necessary.

Since clinical samples are often repeatedly assayed and samples are thawed and refrozen, it is necessary to check for any instability of samples during these processes. Practically, this study can be done by using two concentration samples (High and Low). The same volume of biological fluid used to prepare standard curve samples is aliquoted to the appropriate number of tubes. Samples (in duplicate) are thawed and refrozen (a cycle) for 5 cycles. Samples are repeatedly thawed and refrozen according to the following table. Samples are thawed as if for sample preparation to room temperature and are

left to stand at room temperature for 1 hour.

Cycle	Keep these samples in freezer
1	a
2	a, b
3	a, b, c
4	a, b, c, d
5	a, b, c, d, e

Following Cycle 5, all of the samples are thawed to room temperature and assayed with a standard curve. Test sample concentrations are calculated and reported in a table for each concentration (n=2) of mean concentrations (n=2) at each test point (n=5).

System (processed sample) stability: Stability of drug and internal standard in biological samples prepared for analysis as described above under "Sample Preparation" were demonstrated by assay of sets of control samples with concentrations to cover the standard curve range. Assay results were obtained for prepared samples that were left standing at room temperature for various times after preparation.

Long term stability: Stability of a drug in a biological specimen stored at -20°C and/or -70°C was demonstrated by assay of sets of control samples prepared as described under "Sample Preparation" at concentrations to cover the standard curve range. Long term stability samples were kept at -70°C or -20°C until prepared and analyzed.

Bench top (unprocessed sample) stability: Plasma samples were left to stand at room temperature for various times after generation, then were kept at -70°C until prepared and analyzed.

Purity of Standard Chemicals

The standard chemicals used in a study are USP™ reference standards (if available) or pure chemicals provided by the sponsor, unless otherwise instructed.

A chemical purchased from a general chemical company is not used as a standard, except in unusual circumstances and when its purity can be verified against a USP™ reference standard or the sponsor's standard verified by a certificate of analysis. The internal standard is not under this restriction.

USP™ reference standards can be regarded as 100% pure (unless specified), and no purity correction factor for concentration calculations is necessary. However, a sponsor's standard chemical must be regarded as possibly impure and a correction factor should be considered.

When verifying non USP™ reference standard chemical purity, the working standard solution is run under the method used for assaying biological samples. Each solution is injected 3 times, and two standard solutions are prepared for each standard chemical.

A copy of the supplier's certification sheet is saved with the method validation files.

Routine Assay Procedure

The following are the steps carried out when samples arrive for routine analysis. Sample arrival is recorded in the sample log-in book and on the log-in sheet, which includes the name of the shipper, arrival date, number of samples, sample storage location, and sample condition. An analytical procedure (AP) is normally completed prior to routine analysis. In this AP, the method description is condensed to about 3-5 pages that contain information regarding instrumentation, assay conditions, source of chemicals, preparation of stock solutions, sample preparation and a representative chromatogram.

For routine sample analysis, standard curve, blank and control samples are also analyzed. Sets of equipment consisting of a pump, detector, column, integrator and autosampler were set up for routine assay. Each system is tested by assigning personnel to run a series of controls; the performance of equipment and technical personnel are validated before routine sample assay.

Carry over testing is performed for each run by assay of a blank sample (not spiked with internal standard or with drug standard) immediately following the assay of a high concentration control.

To monitor variation during the course of an assay, a series of controls are prepared beforehand and stored in the freezer. Control samples are run together with standards and routine assay samples. Each set of controls normally includes four different concentrations within the range of the standard curve. For every group, treatment, or up to 20 routine assay samples, a set of controls (e.g. extra low, low, medium and high) is included to validate the results. Since the concentrations of the controls are known, it is possible to judge whether the routine assay samples must be repeated on the basis of the results obtained for the controls.

Assay samples are prepared by spiking known volumes of biological sample with a known amount (constant over all samples) of internal standard (IS). Standard curve samples are generated by spiking interference free biological samples with known amounts of standard compound and IS. These standard curve and assay samples are prepared according to the analytical procedure, then injected onto an HPLC column for separation and subsequent detection. The peak height ratio of standard compound to IS is calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and standard compound to IS peak height ratios of the standard curve samples are fit by weighted or non weighted least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak height ratio and x = standard compound concentration), and standard compound concentrations in assay samples are calculated by this equation from the standard compound to IS peak height ratios obtained by HPLC.

Assay findings are then sent in a report with a complete assay methodology including detailed methods, statistical evaluation of methods, routine assay sample results, results from the control samples, and one representative set of calibration chromatograms. Results can be sent by disc or through a modem for pharmacokinetic evaluation.

EXPERIMENTAL METHODS

The goals of the research under contract DAMD17-92-C-2028 are 1) to develop and validate methods to assay for drug substances in biological fluids for pharmacokinetic, bioavailability, drug metabolism and drug monitoring studies, and 2) to use these methods to perform routine analyses of biological specimens to support pharmacokinetic and bioavailability studies as part of preclinical and clinical investigations undertaken for the purpose of new drug development.

METHOD DEVELOPMENT AND/OR VALIDATION RESULTS

The following section describes the status of specific methods developed and validated or currently being developed and/or validated during the contract.

TABLE 4: STUDY REPORTS

No.	Date/ Status	Report Title	Test Article	Test System	Lower Limit of Quantitation
13	10/28/94 final report	Supplement I: Quantitation of WR 238605 as Free Base in Rat Plasma by HPLC and Fluorescence Detection	WR 238,605	Rat Plasma	2.00 ng/ml
13	4/11/94 in revision	Supplement II: Quantitation of WR 238605 as Free Base in Dog Plasma by HPLC and Fluorescence Detection	WR 238,605	Dog Plasma	1.00 ng/ml
17A	4/25/90 final	Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	halofantrine WR 178,460 halofantrine WR 178,460	Human Plasma Plasma Blood Blood	0.960 ng/ml 0.928 ng/ml 0.960 ng/ml 0.928 ng/ml
17B	12/13/95 final as amended 4/26/96	Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	halofantrine WR 178,460 halofantrine WR 178,460	Human Plasma Plasma Blood Blood	2 ng/ml 2 ng/ml 0.960 ng/ml 0.928 ng/ml
17B	11/1/96 in review	Supplement I: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Perfusate by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Halofantrine WR178460	rat perfusate	0.520 µg/ml 0.510 µg/ml
17B	11/25/96 in review	Supplement II: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Perfusate by Extraction and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Halofantrine WR178460	rat perfusate	10.4 ng/ml 10.2 ng/ml
17B	12/3/96 in review	Supplement III: Quantitation of Halofantrine and WR 178,460 (as Free Basês) in Rat Bile by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Halofantrine WR178460	rat bile	0.416 µg/ml 0.408 µg/ml
17B	12/5/96 in review	Supplement IV: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Bile by Extraction and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Halofantrine WR178460	rat bile	20.4 ng/ml

TABLE 4: STUDY REPORTS

No.	Date/ Status	Report Title	Test Article	Test System	Lower Limit of Quantitation
17B	1/28/97 in review	Supplement V: Quantitation of Halofantrine and WR 178,460 (as Free Bases) in Rat Liver by Precipitation and HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Halofantrine WR178460	rat liver	0.540 µg/ml 0.540 µg/ml
18	7/31/91 status report	Quantitation of WR 6026 and WR 211,789 (WR 6026 Metabolite) in Plasma and Blood by HPLC with a Silica Gel Column and an Aqueous Mobile Phase	WR 6026 WR 211789	Plasma Blood	0.980 ng/ml 1.21 ng/ml
19	1/14/92 in revision	Quantitation of Mefloquine in Human Blood By HPLC, Extraction Method	mefloquine WR 160972	human blood	7.36 ng/ml
20	7/27/94 final report	Quantitation of Artelinic acid in Plasma by HPLC with a C18 Bonded Column	Artelinic Acid	human plasma	4.96 ng/ml
21	Validation complete, report in preparation	Tentative title: Quantitation of *p*-Aminoheptanophenone, *p*-Aminooctanophenone, and *p*-Aminopropiophenone in Dog Plasma by HPLC	PAHP PAPP PAOP	dog plasma	4.08 ng/ml 4.04 ng/ml 4.16 ng/ml
22	7/18/94 in revision	Quantitation of WR 6026, WR 211,789, and WR 254,421 (as Free Bases) in Human Urine By HPLC	WR 6026 WR 211,789 WR 254,421	human urine	5.17 ng/ml 5.09 ng/ml 45.4 ng/ml
23	4/29/96 final report	Quantitation of Primaquine (Free Base) and its Carboxylated Metabolite in Human Plasma by HPLC and Ultraviolet Detection	Primaquine WR 249725	human plasma	28.5 ng/ml 20.0 ng/ml
24	1/7/97 in review	Quantitation of Paromomycin and Gentamicin in Human and Rat Plasma by HPLC	Gentamicin Paromomycin Gentamicin Paromomycin	human plasma rat plasma	0.1 µg/ml 0.1 µg/ml 0.1 µg/ml 0.1 µg/ml

TABLE 4: STUDY REPORTS

No.	Date/ Status	Report Title	Test Article	Test System	Lower Limit of Quantitation
25	11/22/95 final report as amended 3/29/96	Quantitation of Pyridostigmine (as Free Base) in Human Plasma By HPLC with a Silica Gel Column and an Aqueous Mobile Phase	Pyridostigmine	human plasma	1.53 ng/ml
26	12/12/96 final report	Quantitation of WR 242511 (as Free Base) in Human and Dog Plasma By HPLC with a Silica Gel Column and an Aqueous Mobile Phase	WR 242511 WR 242511	human plasma dog plasma	4.00 ng/ml 4.00 ng/ml
27	in development	Tentative title: Quantitation of WR 238605 R&S Separation in Human Plasma by HPLC	WR 238605	human plasma	
28	In development	Tentative title: Quantitation of R&S Isomers of Halofantrine and WR 178,460 in Human Plasma by HPLC	Halofantrine WR 178,460	human plasma	
29	In validation	Tentative title: Quantitation of Chloroquine and Desethyl-chloroquine in Human Plasma by LC/MS/MS	Chloroquine Desethyl-chloroquine	human plasma	
30	In validation	Tentative title: Quantitation of WR 243251 in Human Plasma by LC/MS/MS	WR 243251	human plasma	1 to 5 ng/ml
31	In validation	Tentative title: Quantitation of WR 238,605, Mefloquine, Chloroquine, Quinine and Doxycycline in Dog Plasma by LC/MS/MS	WR 238605 Mefloquine Chloroquine Quinine Doxycycline	dog plasma	
32	In validation	Tentative title: Quantitation of WR 238,605 in Human Plasma by LC/MS/MS	WR 238605	human plasma	
33	In validation	Tentative title: Quantitation of Halofantrine and WR 178,460 in Human Plasma by LC/MS/MS	Halofantrine WR 178,460	human plasma	

Quantitation of WR 238,605 in Human Plasma and Blood and Rat and Dog Plasma

Study Characteristics: Study Report 13B	
Test Article:	WR 238,605
Test System:	human plasma and blood
Internal Standard:	WR 6026
Sample Assay Volume:	0.2 ml
Sample Cleanup:	extract with methyl *t*-butyl ether
Analytical System	
Detector:	Fluorescence with excitation - 375 nm; emission - 480 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5μ particle size
Mobile Phase:	acetonitrile/water (1:1, v/v) final concentration of 5 mM $(NH_4)_2HPO_4$ at pH 7.0.
Validation Results: human plasma	
Quantitation Limit:	0.815 ng/ml
Standard curve range:	0.815-408 ng/ml
Interday Precision	
Concentration Range:	1.63-163 ng/ml
CV Range:	6.64-8.70%
Intraday Precision	
Concentration Range:	1.63-163 ng/ml
CV Range:	5.44-9.07%
Blind Sample Assay	
Concentration Range:	1.20-179.4 ng/ml
Bias Range:	-4.68 to +31.7%
Mean Recovery:	82.5%
Stable Plasma Storage:	-20°C for 4 months
Validation Results (continued): human blood	
Quantitation Limit:	1.91 ng/ml
Standard curve range:	1.91-383 ng/ml
Interday Precision	
Concentration Range:	3.82-143 ng/ml
CV Range:	2.94-7.75%
Intraday Precision	
Concentration Range:	3.82-143 ng/ml
CV Range:	7.69-9.15%
Blind Sample Assay	
Concentration Range:	2.39-239.16 ng/ml
Bias Range:	-22.7 to -5.86%
Mean Recovery:	97.3%
Stable Blood Storage:	-20°C for 30 days

Study Description: WR 238,605 in Human Plasma and Blood

WR 238,605 succinate (N4-[2,6-dimethoxy-4-methyl-5-[(3-trifluoromethyl) phenoxy]-8-quinolinyl]-1,4, pentanediamine succinate), an 8-aminoquinoline derivative, has been chosen by the U.S. Army Drug Development Program to be developed as an anti-malarial drug to replace primaquine.[9] Although primaquine is the best available drug for the curative treatment of *Plasmodium vivax* malaria[10], problems associated with its toxicity[11] and the development of resistant strains,[12] led to the development of WR 238,605 succinate, which was found to be 12.8 times more potent on a molar basis than primaquine in curing the rhesus monkey of *P. cynomolgi*, the simian equivalent of *P. vivax* .[9]

Plasma samples were analyzed for WR 238,605 (free base) (N4-[2,6-dimethoxy-4-methyl-5-[(3-trifluoromethyl)phenoxy]-8-quinolinyl]-1,4, pentanediamine base), an 8-aminoquinoline derivative, with an HPLC procedure that uses a silica gel column, an (acetonitrile/water) aqueous mobile phase, a fluorescence detector, and 0.2 ml plasma samples. Sample cleanup consisted of extraction into methyl *t*-butyl ether, evaporation of the organic phase and reconstitution of plasma sample extracts in acetonitrile/ water prior to separation by HPLC. The attached method SOPs (Appendix A) contains detailed procedures and results, which are summarized below.

Assay samples were prepared by spiking known plasma volumes with a known amount (constant over all samples) of WR 6026 internal standard (IS). Standard curve samples were generated by spiking interference free plasma samples with known amounts of WR 238,605 (free base) and IS. Standard curve and assay samples were extracted, then injected onto an HPLC column for separation and subsequent fluorometric detection. The peak height ratio of WR 238,605 (free base) to IS was calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and WR 238,605 (free base) to IS peak height ratios of the standard curve samples were fit by least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak height ratio and x = WR 238,605 (free base) concentration), and drug concentrations in assay samples were calculated by this equation from the WR 238,605 (free base) to IS peak height ratios obtained by HPLC.

Plasma samples (0.2 ml aliquots unless otherwise specified) to be assayed for WR 238,605 (free base) were pipetted into glass culture tubes. Next, 20 µl of internal standard (WR 6026, 6-methoxy-8-(6-diethylamino-hexylamino lepidine dihydrochloride) solution (27.9 µg/ml) and 0.1 ml of 0.1 N NaOH buffer were added and vortexed. Then, 3 ml of methyl *t*-butyl ether extracting solvent was added, and the samples were vortexed for 1 min, twice, and centrifuged for 10 min at 3000 *g*. The organic layer of each sample was pipetted to a clean tube and evaporated to dryness under nitrogen. The residue was reconstituted with 200 µl of acetonitrile/water (1:1, v/v), transferred to a WISP insert, and injected onto the HPLC column.

Study Characteristics: Short Validation Supplement I to SR 13

Test System:	Rat Plasma
Validation Results:	
Quantitation Limit:	1.00 ng/ml
Precision CV:	6.72%
Precision Error:	21.8%
Standard curve range:	1.00-400 ng/ml
Interday Precision	
Concentration Range:	2.04-204 ng/ml
CV Range:	0.54-6.46%
Intraday Precision	
Concentration Range:	2.04-204 ng/ml
CV Range:	4.62-10.1%
Mean Recovery:	66.5%

Study Description: WR 238,605 in Rat Plasma

The original validation Study Report (No. 13B) for WR 238,605 described validation for human plasma and blood. To satisfy requirements of our current laboratory SOP on validation, a short validation study was performed, Study Report 13B, Supplement I titled "Quantitation of WR 238,605 as Free Base in Rat Plasma by High-Performance Liquid Chromatography and Fluorescence Detection," consisting of precision (a shortened 3 run interday analysis and the normal intraday analysis) and recovery tests with rat plasma.

Study Characteristics: Short Validation Supplement II to SR 13

Test System:	Dog Plasma
Validation Results:	
Quantitation Limit:	1.00 ng/ml
Interday CV:	15.0%
Interday Error:	25.3%
Standard curve range:	1.00-400 ng/ml
Interday Precision	
Concentration Range:	2.04-204 ng/ml
CV Range:	4.60-10.2%
Intraday Precision	
Concentration Range:	2.04-204 ng/ml
CV Range:	1.20-10.8%
Mean Recovery:	66.4%

Study Description: WR 238,605 in Dog Plasma

An additional short validation study, Study Report 13B, Supplement II titled "Quantitation of WR 238,605 as Free Base in Dog Plasma by High-Performance Liquid Chromatography and Fluorescence Detection," with dog plasma was performed.

Halofantrine and Metabolite in Human Plasma and Blood, Rat Liver, Rat Bile and Rat Perfusate

Study Characteristics: Study Report 17

Test Article:	halofantrine, WR 178,460
Test System:	human plasma and blood
Internal Standard:	procainamide hydrochloride
Sample Assay Volume	0.5 ml
Sample Cleanup:	Precipitate with acetonitrile extract with methyl *t*-butyl ether

Analytical System

Detector:	fluorescence
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	CH_3OH/water (80:20, v/v) 5 mM $(NH_4)_2HPO_4$

Validation Results: halofantrine in human plasma

Quantitation Limit:	0.960 ng/ml
Standard curve range:	0.960-115 ng/ml
Interday Precision	
Concentration Range:	1.92-76.8 ng/ml
CV Range:	4.24-10.6%
Intraday Precision	
Concentration Range:	1.92-76.8 ng/ml
CV Range:	3.82-13.6%
Mean Recovery:	73.0%
Stable Plasma Storage:	-80°C for 4 months

Validation Results: WR 178,460 in human plasma

Quantitation Limit:	0.928 ng/ml
Standard curve range:	0.928-111 ng/ml
Interday Precision	
Concentration Range:	1.86-74.2 ng/ml
CV Range:	4.90-6.65%
Intraday Precision	
Concentration Range:	1.86-74.2 ng/ml
CV Range:	4.21-7.94%
Mean Recovery:	95.5%
Stable Plasma Storage:	-80°C for 4 months

Validation Results: halofantrine in human blood

Quantitation Limit:	0.960 ng/ml
Standard curve range:	0.960-115 ng/ml
Interday Precision	
Concentration Range:	1.92-76.8 ng/ml
CV Range:	3.42-10.5%
Intraday Precision	
Concentration Range:	1.88-75.4 ng/ml
CV Range:	4.60-10.0%
Mean Recovery:	68.6%
Stable Blood Storage:	-80°C for 4 months

Validation Results: WR 178,460 in human blood

Quantitation Limit:	0.928 ng/ml
Standard curve range:	0.928-111 ng/ml
Interday Precision	
Concentration Range:	1.86-74.2 ng/ml
CV Range:	6.49-7.88%
Intraday Precision	
Concentration Range:	1.85-73.9 ng/ml
CV Range:	5.70-9.40%
Mean Recovery:	95.9%
Stable Blood Storage:	-80°C for 4 months

Study Description: Halofantrine and Metabolite in Human Plasma and Blood

Study Report 17 presents a second approach to the HPLC analysis of blood and plasma samples for determination of the free base concentrations of halofantrine (WR 171,669) and of its metabolite (WR 178,460). Study Report No. 4 (dated August 23, 1985 under contract DAMD 17-83-C-3004) describes an ion-paired liquid chromatographic assay for halofantrine and its metabolite as free bases in blood & plasma. The assay involves protein precipitation and a column elution step prior to HPLC separation. However, due to successes in this laboratory in assays for several amines in which a silica gel stationary phase was used[13,14] the same approach was tried in an assay for halofantrine and its metabolite (as free bases). The second method involves the use of a silica gel column run with an aqueous mobile phase which results in cleaner baselines and a higher signal to noise ratio at concentrations similar to the ion-paired method.[15] All glassware used in this assay must be silanized to limit error due to absorption.

Plasma samples for analysis are pipetted (0.5 ml) into silanized tubes. Approximately 1.5 ng of the internal standard, procainamide hydrochloride,

and 1 ml of CH_3CN to precipitate the proteins were added. The samples are vortexed and centrifuged and the resulting supernatant is transferred to a silanized tube and evaporated under N_2 to about 0.5 ml. Water (0.5 ml) and 0.1 N NaOH buffer (0.5 ml) were added to make the solution alkaline. Methyl *t*-butyl ether (5 ml), the extracting solvent, is added and the sample is vortexed, centrifuged and frozen in a dry ice/MeOH bath. The organic layer is poured into a 13x100 mm silanized tube. This extraction step is repeated with another 5 ml of extracting solvent which is poured into the same 13x100 mm silanized tube. The solvent is evaporated to dryness under N_2, the residue is reconstituted with 200 µl of methanol/water (80:20) containing 0.001% HCl, and 25 to 150 µl is injected onto the column.

Blood samples are treated similarly except 0.5 ml water to lyse the cells and 2 ml of CH_3CN to precipitate the proteins were added. For standard curve samples, appropriate amounts of drug and metabolite are added, and samples are left to equilibrate at room temperature for one hour.

A silica gel column and a mobile phase composition of methanol/water (80:20, v/v) with a final concentration 5 mM $(NH_4)_2HPO_4$ were used to separate halofantrine and WR 178,460 (as free bases) from the internal standard and interfering endogenous substances in an isocratic elution. In typical chromatograms, halofantrine and WR 178,460 (as free bases) for both plasma and blood are sufficiently separated from each other and from endogenous compounds to permit successful sample assay. Halofantrine (free base) eluted at 8 minutes, WR 178,460 (free base) eluted at 11 minutes, and the internal standard eluted at 26 minutes.

A linear relationship was demonstrated between the halofantrine and WR 178,460 (as free bases) concentrations in plasma or blood and the peak height ratios of the halofantrine or WR 178,460 peak to the internal standard peak. The minimum detection limits, 0.928 and 0.960 ng/ml, were determined as the halofantrine and WR 178,460 (as free bases) concentrations, respectively, at which the signal to noise ratio was 3 to 1. Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level drug or metabolite (as free bases) concentrations, two standard curves were constructed from the same set of standard curve data points.

Data points from 0 to 14.4 ng/ml were used to construct a low halofantrine (free base) concentration standard curve with which to calculate the low concentration samples. All data points (0 to 115 ng/ml) were used to construct a high halofantrine (free base) concentration standard curve for the high concentration samples. Concentrations of samples with peak height ratios above that calculated at 14.4 ng/ml from the low concentration standard curve were calculated with the high concentration standard curve.

Data points from 0 to 13.9 ng/ml were used to construct a low WR 178,460 (free base) concentration standard curve with which to calculate the low

concentration samples. All data points (0 to 111 ng/ml) were used to construct a high WR 178,460 (free base) concentration standard curve for the high concentration samples. Concentrations of samples with peak height ratios above that calculated at 13.9 ng/ml from the low concentration standard curve were calculated with the high concentration standard curve.

For representative standard curves, the coefficients of determination for halofantrine (free base) in plasma were 0.9995 and 0.9950 and for halofantrine (free base) in blood were 0.9985 and 0.9985 for low and high concentration ranges, respectively. The coefficients for WR 178,460 (free base) in plasma were 0.9998 and 0.9983 and for WR 178,460 (free base) in blood were 0.9956 and 0.9993 for low and high concentration ranges, respectively.

Blind blood samples, prepared April 1, 1993 were assayed by the method described in Study Report 17, modified by incorporation of an analog to halofantrine (WR 122,455) as an alternate internal standard. The results, and changes induced by using a different internal standard, will be incorporated in Study Report No. 17B.

Study Characteristics: Study Report 17B

Test Article:	halofantrine, WR 178,460
Test System:	human plasma and blood
Internal Standard:	WR 122,455
Sample Assay Volume:	0.5 ml
Sample Cleanup:	Precipitate with acetonitrile Double extraction with methyl *t*-butyl ether

Analytical System

Detector:	fluorescence-Ex: 300 nm, Em: 375 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	CH_3OH/water (80:20, v/v) 5 mM $(NH_4)_2HPO_4$

Validation Results: halofantrine in human plasma

Lower Limit of Quantitation:	2.08 ng/ml
Interday Mean, CV and RE:	1.81 ng/ml, 12.0% and -13.0%
Intraday Mean, CV and RE:	1.97 ng/ml, 18.2% and -5.67%
Standard curve range:	2.08 to 266 ng/ml
Interday Precision Concentrations:	4.04, 10.1, 40.4, and 129 ng/ml
CV Range:	7.17 to 14.2%
RE Range:	-1.87 to +3.31%
Intraday Precision Concentrations:	2.04, 10.2, 61.2, and 102 ng/ml
CV Range:	3.83 to 8.75%
RE Range:	-16.3 to -1.00%
Blind Sample Assay	Run in conjunction with Study Report 17
Overall Mean Recovery:	46.4%

Stability

Plasma Freezer Storage:	-80°C for 4 months
Processed Sample:	Room temp. for 1 day
Plasma Storage:	On ice for 4 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Validation Results: WR 178,460 in human plasma

Lower Limit of Quantitation:	2.08 ng/ml
Interday Mean, CV and RE:	2.02 ng/ml, 13.3% and -3.06%
Intraday Mean, CV and RE:	2.11 ng/ml, 4.07% and +1.52%
Standard curve range:	2.08 to 266 ng/ml
Interday Precision Concentrations:	4.16, 10.4, 41.6, and 133 ng/ml
CV Range:	4.19 to 12.3%
RE Range:	-0.149 to +3.91%
Intraday Precision Concentrations:	1.93, 9.64, 57.9, and 96.5 ng/ml
CV Range:	1.62 to 6.95%
RE Range:	-3.49 to +6.11%
Blind Sample Assay	Run in conjunction with Study Report 17
Overall Mean Recovery:	80.9%
Stability	
Plasma Freezer Storage:	-80°C for 4 months
Processed Sample:	Room temp. for 1 day
Plasma Storage:	On ice for 4 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Validation Results: halofantrine in human blood

Lower Limit of Quantitation:	1.02 ng/ml
Interday Mean, CV and RE:	1.14 ng/ml, 12.2% and +11.4%
Intraday Mean, CV and RE:	1.12 ng/ml, 10.1% and +9.45%
Standard curve range:	1.02-245 ng/ml
Interday Precision Concentrations:	4.16, 10.4, 41.6, and 133 ng/ml
CV Range:	5.38 to 10.8%
RE Range:	-3.12 to +5.08%
Intraday Precision Concentrations:	2.04, 10.2, 61.2, and 102 ng/ml
CV Range:	9.58 to 16.7%
RE Range:	-9.31 to +0%
Blind Sample Assay	
Concentration Range:	2.04 to 183.6 ng/ml
RE Range:	-8.21 to +3.68%
Overall Mean Recovery:	80.8%
Stability	
Plasma Freezer Storage:	-80°C for 4 months
Processed Sample:	Room temp. for 1 day
Plasma Storage:	On ice for 4 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Validation Results: WR 178,460 in human blood

Lower Limit of Quantitation:	0.964 ng/ml
Interday Mean, CV and RE:	0.980 ng/ml, 6.35% and +1.70%
Intraday Mean, CV and RE:	0.923 ng/ml, 9.57% and -4.28%
Standard curve range:	0.964 to 232 ng/ml
Interday Precision Concentrations:	1.93, 9.64, 57.9, and 96.5 ng/ml
CV Range:	2.90 to 7.67%
RE Range:	-4.04 to -0.0765%
Intraday Precision Concentrations:	2.04, 10.2, 61.2, and 102 ng/ml
CV Range:	3.26 to 4.33%
RE Range:	-5.34 to +0.400%
Blind Sample Assay	
Concentration Range:	1.97 to 177.5 ng/ml
RE Range:	-2.47 to +33.8%
Overall Mean Recovery:	90.4%
Stability	
Plasma Freezer Storage:	-80°C for 4 months
Processed Sample:	Room temp. for 1 day
Plasma Storage:	On ice for 4 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Study Description: Halofantrine and Metabolite in Human Plasma and Blood

The original version (Study Report 17 dated 4/25/90) of the silica gel stationary phase method has been modified by replacement of procainamide with WR 122,455 as internal standard and by the additional requirement that sample preparation is performed over ice.

Study Characteristics: Study Report 17B, Supplement I

Sample Assay Volume:	0.1 ml
Sample Cleanup:	Precipitate with acetonitrile

Short Validation Results: Halofantrine as free base in rat perfusate by precipitation

Lower Limit of Quantitation:	0.520 µg/ml
Interday Mean, CV and RE:	0.553 µg/ml, 2.35% and +6.35%
Intraday Mean, CV and RE:	0.502 µg/ml, 9.90% and -3.56%
Standard curve range:	0.520 to 33.28 µg/ml
Interday Precision Concentrations:	1.18, 5.90, and 2.36 µg/ml
CV Range:	1.36 to 4.09%
RE Range:	-4.72 to -0.141%
Intraday Precision Concentrations:	1.18, 5.90, and 2.36 µg/ml
CV Range:	1.60 to 3.55%
RE Range:	-5.28 to -2.90%
Overall Mean Recovery:	99.1%

Short Validation Results: WR 178460 as free base in rat perfusate by precipitation

Lower Limit of Quantitation:	0.510 µg/ml
Interday Mean, CV and RE:	0.555 µg/ml, 0.480% and +8.82%
Intraday Mean, CV and RE:	0.558 µg/ml, 8.29% and +9.38%
Standard curve range:	0.510 to 33.64 µg/ml
Interday Precision Concentrations:	1.07, 5.35, and 20.4 µg/ml
CV Range:	2.40 to 3.61%
RE Range:	-4.92 to +4.49%
Intraday Precision Concentrations:	1.07, 5.35, and 20.4 µg/ml
CV Range:	2.04 to 3.40%
RE Range:	-5.02 to +3.68%
Overall Mean Recovery:	100%
Sample Assay Volume:	0.5 ml

Study Description: Halofantrine and Metabolite in Rat Perfusate by Precipitation

The perfusate samples were analyzed for halofantrine (1,3-dichloro-6-trifluoromethyl-9-[1-hydroxy-3-(di-*n*-butylaminopropyl)-phenanthrene hydrochloride) and WR 178,460 (1,3 dichloro-6-trifluoromethyl-9-[1-hydroxyl-3-(N-*n*-butylamino)propyl]-phenanthrene hydrochloride) (as free bases) with an HPLC procedure that uses a silica gel column, a (methanol/water) aqueous mobile phase, a fluorescence detector, and 100 µl rat perfusate samples.

Sample cleanup consisted of precipitation and centrifugation of perfusate samples with acetonitrile prior to separation by HPLC.

Assay samples were prepared by spiking known volumes of rat perfusate with a known amount (constant over all samples) of WR 122,455 (α-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride) internal standard (IS). Standard curve samples were generated by spiking interference free rat perfusate samples with known amounts of halofantrine and WR 178,460 (as free bases) and IS. These standard curve and assay samples were precipitated and centrifuged, then injected onto an HPLC column for separation and subsequent fluorometric detection. The peak height ratios of halofantrine and WR 178,460 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and halofantrine and WR 178,460 (as free bases) to IS peak height ratios of the standard curve samples were fit by weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$, where y = peak height ratio and x = halofantrine or WR 178,460 (as free bases) concentration), and drug and metabolite concentrations in assay samples were calculated by these equations from the halofantrine and WR 178,460 (as free bases) to IS peak height ratios obtained by HPLC.

Rat perfusate samples (100 µl aliquots unless otherwise specified) to be assayed for halofantrine and WR 178,460 (as free bases) were pipetted into silanized glass culture tubes. Next, 40 µl of internal standard (WR 122,455) solution (9.90 µg/ml) was added to each and the samples vortexed. Then, 0.4 ml of acetonitrile precipitant was added to each, and the samples were vortexed for 1 min. and centrifuged for 10 min. at 3000 *g*. Each supernatant was transferred to a silanized WISP insert and injected onto the HPLC column.

By use of simple precipitation and centrifugation for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of fluorescence detection, halofantrine and WR 178,460 (as free bases) can be quantitatively and reliably measured in rat perfusate samples. The assay described in the report dated November, 11, 1996, requires 100 µl rat perfusate samples to determine the concentrations in the range 0.520 - 33.28 µg/ml halofantrine (as free base) and 0.510 - 32.64 µg/ml WR 178,460 (as free base). The method involves sample cleanup with acetonitrile precipitation, separation on a silica column run with an aqueous mobile phase, and fluorescence detection. Retention times and peak shapes do not appreciably change in this particular assay within the specified injection volumes (5-20 µl) as can be seen by examination of the representative standard curve chromatograms and the corresponding standard curve (Table 1). In some assays, such changes are appreciable and can adversely affect the standard curve, requiring identical injection volumes for all samples. The LLOQs for the assay of rat perfusate, based on interday

and intraday low point validation results (Table 2), has been set at 0.520 µg/ml for halofantrine and 0.510 µg/ml for WR 178,460 (as free bases). The average mean recoveries over the working range of the methodology were 99.1% for halofantrine and 100% for WR 178,460 (as free bases). The interday and intraday precision C.V.'s ranged from 1.36 to 4.09% and 1.60 to 3.55% for the method, respectively, for halofantrine and from 2.40 to 3.61% and 2.04 to 3.40% for the method, respectively, for WR 178,460 (as free bases).

Study Characteristics: Study Report 17B, Supplement II

Sample Assay Volume:	0.1 ml
Sample Cleanup:	Double extraction with methyl *t*-butyl ether

Short Validation Results: Halofantrine as free base in rat perfusate by extraction

Lower Limit of Quantitation:	10.4 ng/ml
Interday Mean, CV and RE:	11.3 ng/ml, 6.66% and +8.33%
Intraday Mean, CV and RE:	11.4 ng/ml, 6.30% and +9.78%
Standard curve range:	10.4 to 1331 ng/ml
Interday Precision Concentrations:	23.6, 70.8, 283.2, and 944 ng/ml
CV Range:	4.58 to 7.75%
RE Range:	-9.83 to -2.01%
Intraday Precision Concentrations:	23.6, 70.8, 283.2, and 944 ng/ml
CV Range:	1.49 to 7.12%
RE Range:	-5.72 to +0.140%
Overall Mean Recovery:	90.8%

Short Validation Results: WR 178460 as free base in rat perfusate by extraction

Lower Limit of Quantitation:	10.2 ng/ml
Interday Mean, CV and RE:	10.4 ng/ml, 10.7% and +1.57%
Intraday Mean, CV and RE:	10.7 ng/ml, 7.80% and +4.61%
Standard curve range:	10.2 to 1306 ng/ml
Interday Precision Concentrations:	21.4, 64.2, 257, and 856 ng/ml
CV Range:	4.13 to 7.88%
RE Range:	-4.11 to +1.78%
Intraday Precision Concentrations:	21.4, 64.2, 257, and 856 ng/ml
CV Range:	0.68 to 8.99%
RE Range:	-5.45 to +4.36%
Overall Mean Recovery:	92.4%

Study Description: Halofantrine and Metabolite in Rat Perfusate by Extraction

The perfusate samples were analyzed for halofantrine (1,3-dichloro-6-trifluoromethyl-9-[1-hydroxy-3-(di-*n*-butylaminopropyl)-phenanthrene hydrochloride) and WR 178,460 (as free bases) (1,3 dichloro-6-trifluoromethyl-9-[1-hydroxyl-3-(N-*n*-butylamino)propyl]-phenanthrene hydrochloride), with an HPLC procedure that uses a silica gel column, a (methanol/water) aqueous mobile phase, a fluorescence detector, and 100 µl rat perfusate samples. Sample cleanup consisted of extraction into methyl *t*-butyl ether, evaporation of extracted samples and reconstitution in methanol/0.001% HCl (4:1, v/v)

prior to separation by HPLC. The attached method SOP (Appendix A) contains detailed procedures and results, which are summarized below.

Assay samples were prepared by spiking known volumes of rat perfusate with a known amount (constant over all samples) of WR 122,455 (α-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride) internal standard (IS). Standard curve samples were generated by spiking interference free rat perfusate samples with known amounts of halofantrine and WR 178,460 (as free bases) and IS. These standard curve and assay samples were extracted, the extracts evaporated to dryness and reconstituted, then injected onto an HPLC column for separation and subsequent fluorometric detection. The peak height ratios of halofantrine and WR 178,460 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and halofantrine and WR 178,460 (as free bases) to IS peak height ratios of the standard curve samples were fit by weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$, where y = peak height ratio and x = halofantrine or WR 178,460 (as free bases) concentrations), and drug and metabolite concentrations in assay samples were calculated by this equation from the halofantrine and WR 178,460 (as free bases) to IS peak height ratios obtained by HPLC.

Rat perfusate samples (100 µl aliquots unless otherwise specified) to be assayed for halofantrine and WR 178,460 (as free bases) were pipetted into 16x125 silanized glass culture tubes. Then, 100 µl water was added. Next, 20 µl of internal standard (WR 122,455) solution (1.04 µg/ml) and 50 µl of 0.1 N NaOH were added and vortexed. Then, 2 ml of methyl *t*-butyl ether was added to each, the samples were vortexed for 1 min, twice, and centrifuged for 10 min at 3000 *g*. Each samples' aqueous layer was frozen in a dry ice/methanol bath and the extraction solvent transferred to a 13x100 silanized tube. The extraction was repeated. Combined extracts were evaporated to dryness, reconstituted in 200 µl methanol/water (4:1, v/v) with a final 0.001% HCl concentration, and samples were transferred to silanized WISP inserts, and injected onto the HPLC column.

By use of a simple double extraction and centrifugation for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of fluorescence detection, halofantrine and WR 178,460 (as free bases) can be quantitatively and reliably measured in rat perfusate samples. The assay described in the report dated November, 25, 1996, requires 100 µl rat perfusate samples to determine the concentrations in the range 10.4 - 1331 ng/ml halofantrine (as free base) and 10.2 - 1306 ng/ml WR 178,460 (as free base). The method involves sample extraction (double) into methyl *t*-butyl ether, separation on a silica column run with an aqueous mobile phase, and fluorescence detection. Retention times and peak shapes do not appreciably change in this particular assay

within the specified injection volumes (50-100 µl) as can be seen by examination of the representative standard curve chromatograms and the corresponding standard curve (Table 1). In some assays, such changes are appreciable and can adversely affect the standard curve, requiring identical injection volumes for all samples. The LLOQs for the assay of rat perfusate, based on interday and intraday low point validation results, has been set at 10.4 ng/ml for halofantrine and 10.2 ng/ml for WR 178,460 (as free bases). The average mean recoveries over the working range of the methodology were 90.8% for halofantrine and 92.4% for WR 178,460 (as free bases). The interday and intraday precision C.V.'s ranged from 4.58 to 7.75% and 1.49 to 7.12% for the method, respectively, for halofantrine and from 4.13 to 7.88% and 0.680 to 8.99% for the method, respectively, for WR 178,460 (as free bases). The interday and intraday precision R.E.'s ranged from -9.83 to -2.01% and -5.72 to +0.140% for the method, respectively, for halofantrine and from -4.11 to +1.78% and -5.45 to +4.36% for the method, respectively, for WR 178,460 (as free bases).

Study Characteristics: Study Report 17B, Supplement III

Sample Assay Volume:	25 µl
Sample Cleanup:	Precipitate with acetonitrile

Short Validation Results: Halofantrine as free base in rat bile by precipitation

Lower Limit of Quantitation:	0.416 µg/ml
Interday Mean, CV and RE:	0.463 µg/ml, 1.69% and +11.3%
Intraday Mean, CV and RE:	0.477 µg/ml, 9.64% and +14.6%
Standard curve range:	0.416 to 66.56 µg/ml
Interday Precision Concentrations:	0.944, 4.72, 14.16, and 28.32 µg/ml
CV Range:	2.63 to 6.85%
RE Range:	-7.59 to +2.21%
Intraday Precision Concentrations:	0.944, 4.72, 14.16, and 28.32 µg/ml
CV Range:	1.85 to 3.65%
RE Range:	-7.13 to +3.09%
Overall Mean Recovery:	104%

Short Validation Results: WR 178460 as free base in rat bile by precipitation

Lower Limit of Quantitation:	0.408 µg/ml
Interday Mean, CV and RE:	0.483 µg/ml, 3.05% and +18.4%
Intraday Mean, CV and RE:	0.468 µg/ml, 7.01% and +14.7%
Standard curve range:	0.408 to 65.28 µg/ml
Interday Precision Concentrations:	0.860, 4.30, 12.84, and 25.68 µg/ml
CV Range:	2.50 to 11.6%
RE Range:	-5.41 to +6.29%
Intraday Precision Concentrations:	0.856, 4.28, 12.84, and 25.68 µg/ml
CV Range:	1.28 to 4.12%
RE Range:	-6.07 to +1.85%
Overall Mean Recovery:	103%

Study Description: Halofantrine and Metabolite in Rat Bile by Precipitation

Rat bile samples were analyzed for halofantrine (1,3-dichloro-6-trifluoro-methyl-9-[1-hydroxy-3-(di-*n*-butylaminopropyl)-phenanthrene hydrochloride) and WR 178,460 (as free bases) (1,3 dichloro-6-trifluoromethyl-9-[1-hydroxyl-3-(N-*n*-butylamino)propyl]-phenanthrene hydrochloride), with an HPLC procedure that uses a silica gel column, a (methanol/water) aqueous mobile phase, a fluorescence detector, and 25 µl rat bile samples. Sample cleanup consisted of precipitation and centrifugation of bile samples with acetonitrile prior to separation by HPLC. The attached method SOP (Appendix A) contains detailed procedures and results, which are summarized below.

Assay samples were prepared by spiking known volumes of rat bile with a known amount (constant over all samples) of WR 122,455 (a-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride) internal standard (IS). Standard curve samples were generated by spiking interference free rat bile samples with known amounts of halofantrine and WR 178,460 (as free bases) and IS. These standard curve and assay samples were precipitated and centrifuged, then injected onto an HPLC column for separation and subsequent fluorometric detection. The peak height ratios of halofantrine and WR 178,460 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and halofantrine and WR 178,460 (as free bases) to IS peak height ratios of the standard curve samples were fit by weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$, where y = peak height ratio and x = halofantrine or WR 178,460 (as free bases) concentrations), and drug and metabolite concentrations in assay samples were calculated by this equation from the halofantrine and WR 178,460 (as free bases) to IS peak height ratios obtained by HPLC.

Rat bile samples (25 µl aliquots unless otherwise specified) to be assayed for halofantrine and WR 178,460 (as free bases) were pipetted into silanized glass culture tubes. Next, 10 µl of internal standard (WR 122,455) solution (9.90 µg/ml) was added and each sample was vortexed. Then, 0.2 ml of acetonitrile precipitant was added, and the samples were vortexed for 1 min. and centrifuged for 10 min. at 3000 *g*. The supernatant was transferred to a silanized WISP insert, and 20 µl was injected onto the HPLC column.

By use of simple precipitation and centrifugation for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of fluorescence detection, halofantrine and WR 178,460 (as free bases) can be quantitatively and reliably measured in rat bile samples. The assay described in the report dated December, 3, 1996, requires 25 µl rat bile samples to determine the concentrations in the range 0.416 - 66.56 µg/ml halofantrine (as free base) and 0.408 - 65.28 µg/ml WR 178,460 (as free base). The method involves sample cleanup with acetonitrile precipitation, separation on a silica column run with an aqueous mobile phase, and fluorescence detection. The LLOQs for the assay of rat bile, based on interday and intraday low point validation results, has been set at 0.416 µg/ml for halofantrine and 0.408 µg/ml for WR 178,460 (as free bases). The overall mean recoveries over the working range of the methodology were 104% for halofantrine and 103% for WR 178,460 (as free bases). The interday and intraday precision C.V.'s ranged from 2.63 to 6.85% and 1.85 to 3.65% for the method, respectively, for halofantrine and from 2.50 to 11.6% and 1.28 to 4.12% for the method, respectively, for WR 178,460 (as free bases). The interday and intraday precision R.E.'s ranged from -7.59 to +2.21% and -7.13 to +3.09% for the method, respectively, for halofantrine and from -5.41 to +6.29% and -6.07 to +1.85% for the method, respectively, for WR 178,460 (as free bases).

Study Characteristics: Study Report 17B, Supplement IV

Sample Assay Volume:	0.1 ml
Sample Cleanup:	Double extraction with methyl *t*-butyl ether

Short Validation Results: Halofantrine as free base in rat bile by extraction

Lower Limit of Quantitation:	20.8 ng/ml
Interday Mean, CV and RE:	22.8 ng/ml, 0.62% and +9.62%
Intraday Mean, CV and RE:	23.0 ng/ml, 4.19% and +10.5%
Standard curve range:	20.8 to 1331 ng/ml
Interday Precision Concentrations:	47.2, 94.4, 378, and 944 ng/ml
CV Range:	3.57 to 15.5%
RE Range:	-9.56 to +1.52%
Intraday Precision Concentrations:	47.2, 94.4, 378, and 944 ng/ml
CV Range:	3.82 to 13.4%
RE Range:	-10.3 to -1.45%
Overall Mean Recovery:	70.5%

Short Validation Results: WR 178460 as free base in rat bile by extraction

Lower Limit of Quantitation:	20.4 ng/ml
Interday Mean, CV and RE:	22.6 ng/ml, 7.84% and +10.5%
Intraday Mean, CV and RE:	20.5 ng/ml, 4.63% and +0.69%
Standard curve range:	20.4 to 1306 ng/ml
Interday Precision Concentrations:	42.8, 85.6, 342, and 856 ng/ml
CV Range:	1.70 to 17.0%
RE Range:	-5.67 to -1.01%
Intraday Precision Concentrations:	42.8, 85.6, 342, and 856 ng/ml
CV Range:	2.63 to 11.5%
RE Range:	-3.37 to +4.75%
Overall Mean Recovery:	90.1%

Study Description: Halofantrine and Metabolite in Rat Bile by Extraction

The bile samples were analyzed for halofantrine (1,3-dichloro-6-trifluoromethyl-9-[1-hydroxy-3-(di-*n*-butylaminopropyl)-phenanthrene hydrochloride) and WR 178,460 (as free bases) (1,3 dichloro-6-trifluoromethyl-9-[1-hydroxyl-3-(N-*n*-butylamino)propyl]-phenanthrene hydrochloride), with an HPLC procedure that uses a silica gel column, a (methanol/water) aqueous mobile phase, a fluorescence detector, and 100 µl rat bile samples. Sample cleanup consisted of extraction into methyl *t*-butyl ether, evaporation of extracted samples and reconstitution in methanol/0.001% HCl (4:1, v/v) prior to separation by HPLC. The attached method SOP (Appendix A) contains detailed procedures and results, which are summarized below.

Assay samples were prepared by spiking known volumes of rat bile with a known amount (constant over all samples) of WR 122,455 (a-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride) internal standard (IS). Standard curve samples were generated by spiking interference free rat bile samples with known amounts of halofantrine and WR 178,460 (as free bases) and IS. These standard curve and assay samples were extracted, the extracts evaporated to dryness and reconstituted, then injected onto an HPLC column for separation and subsequent fluorometric detection. The peak height ratios of halofantrine and WR 178,460 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and halofantrine and WR 178,460 (as free bases) to IS peak height ratios of the standard curve samples were fit by weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$, where y = peak height ratio and x = halofantrine or WR 178,460 (as free bases) concentrations), and drug and metabolite concentrations in assay samples were calculated by this equation from the halofantrine and WR 178,460 (as free bases) to IS peak height ratios obtained by HPLC.

Rat bile samples (100 µl aliquots unless otherwise specified) to be assayed for halofantrine and WR 178,460 (as free bases) were pipetted into 16x125 silanized glass culture tubes on ice. Then, 100 µl of water was added. Next, 20 µl of internal standard (WR 122,455) solution (1.04 µg/ml) and 50 µl of 0.1 N NaOH were added and vortexed. Then, 2 ml of methyl *t*-butyl ether was added to each, and the samples were vortexed for 1 min., twice, and centrifuged for 10 min. at 3000 *g*. Each samples aqueous layer was frozen in a dry ice/methanol bath and the extraction solvent transferred to a 13x100 silanized tube. The extraction was repeated. Combined extracts were evaporated to dryness, reconstituted in 200 µl methanol/water (4:1, v/v) with a final 0.001% HCl concentration, and samples were transferred to silanized WISP inserts, and injected onto the HPLC column.

By use of a simple double extraction and centrifugation for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of fluorescence detection, halofantrine and WR 178,460 (as free bases) can be quantitatively and reliably measured in rat bile samples. The assay described in the report dated December, 5, 1996, requires 100 µl rat bile samples to determine the concentrations in the range 20.8 - 1331 ng/ml halofantrine (as free base) and 20.4 - 1306 ng/ml WR 178,460 (as free base). The method involves sample extraction (double) into methyl *t*-butyl ether, separation on a silica column run with an aqueous mobile phase, and fluorescence detection. Retention times and peak shapes do not appreciably change in this particular assay within the specified injection volumes (50-100 µl) as can be seen by examination of the representative standard curve chromatograms and the corresponding standard curve (Table 1). In some assays, such changes are appreciable and can adversely affect the standard curve, requiring identical

injection volumes for all samples. The LLOQs for the assay of rat bile, based on interday and intraday low point validation results, has been set at 20.8 ng/ml for halofantrine and 20.4 ng/ml for WR 178,460 (as free bases). The overall average recoveries over the working range of the methodology were 70.5% for halofantrine and 90.1% for WR 178,460 (as free bases). The interday and intraday precision C.V.'s ranged from 3.57 to 15.5% and 3.82 to 13.4% for the method, respectively, for halofantrine and from 1.70 to 17.0% and 2.63 to 11.5% for the method, respectively, for WR 178,460 (as free bases). The interday and intraday precision R.E.'s ranged from -9.56 to +1.52% and -10.3 to -1.45% for the method, respectively, for halofantrine and from -5.67 to -1.01% and -3.37 to +4.75% for the method, respectively, for WR 178,460 (as free bases).

Study Characteristics: Study Report 17B, Supplement V

Sample Assay Volume:	0.200 ml
Sample Cleanup:	Precipitate with acetonitrile

Short Validation Results: Halofantrine as free base in rat liver homogenate

Lower Limit of Quantitation:	0.540 µg/ml
Interday Mean, CV and RE:	0.584 µg/ml, 9.59% and +8.21%
Intraday Mean, CV and RE:	0.550 µg/ml, 9.30% and +1.76%
Standard curve range:	0.540 to 69.1 µg/ml
Interday Precision Concentrations:	1.07, 5.35, and 24.6 µg/ml
CV Range:	2.52 to 5.45%
RE Range:	+0.187 to +5.62%
Intraday Precision Concentrations:	1.07, 5.35, and 24.6 µg/ml
CV Range:	2.33 to 4.86%
RE Range:	-0.685 to +10.4%
Overall Mean Recovery:	99.1%

Short Validation Results: WR 178460 as free base in rat liver homogenate

Lower Limit of Quantitation:	0.540 µg/ml
Interday Mean, CV and RE:	0.559 µg/ml, 9.11% and +3.58%
Intraday Mean, CV and RE:	0.563 µg/ml, 7.88% and +4.32%
Standard curve range:	0.540 to 69.1 µg/ml
Interday Precision Concentrations:	1.07, 5.35, and 24.6 µg/ml
CV Range:	2.30 to 8.55%
RE Range:	-3.93 to +4.70%
Intraday Precision Concentrations:	1.07, 5.35, and 24.6 µg/ml
CV Range:	2.53 to 5.25%
RE Range:	-2.78 to +5.76%
Overall Mean Recovery:	94.6%

Study Description: Halofantrine and Metabolite in Rat Liver Homogenate

Rat liver samples were analyzed for halofantrine (1,3-dichloro-6-trifluoro-methyl-9-[1-hydroxy-3-(di-*n*-butylaminopropyl)-phenanthrene hydrochloride) and WR 178,460 (as free bases) (1,3 dichloro-6-trifluoromethyl-9-[1-hydroxyl-3-(N-*n*-butylamino)propyl]-phenanthrene hydrochloride), with an HPLC procedure that uses a silica gel column, a (methanol/water) aqueous mobile phase, a fluorescence detector, and 200 µl rat liver homogenate samples. The attached method SOP (Appendix A) contains detailed procedures and results, which are summarized below.

Assay samples were prepared by spiking known volumes of rat liver homogenate with a known amount (constant over all samples) of WR

122,455 (a-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride) internal standard (IS). Liver homogenate is produced by homogenizing 1 g liver in 5 ml of a methanol/water (with 1% final HCl concentration) buffer in a blender. Standard curve samples were generated by spiking interference free rat liver homogenate samples with known amounts of halofantrine and WR 178,460 (as free bases) and IS. These standard curve and assay samples were precipitated and centrifuged, then injected onto an HPLC column for separation and subsequent fluorometric detection. The peak height ratios of halofantrine and WR 178,460 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, spiked concentrations and halofantrine and WR 178,460 (as free bases) to IS peak height ratios of the standard curve samples were fit by weighted least squares linear regression to the equations for the best straight lines ($y = mx + b$, where y = peak height ratio and x = halofantrine or WR 178,460 (as free bases) concentrations), and drug and metabolite concentrations in assay samples were calculated by this equation from the halofantrine and WR 178,460 (as free bases) to IS peak height ratios obtained by HPLC.

Rat liver homogenate samples (200 µl aliquots unless otherwise specified) to be assayed for halofantrine and WR 178,460 (as free bases) were pipetted into silanized glass culture tubes. Then, 0.2 ml of acetonitrile precipitant was added, and the samples were vortexed for 1 min. Next, 40 µl of internal standard solution (9.90 µg/ml WR 122,455) was added and each sample was vortexed and centrifuged for 10 min. at 3000 *g*. The supernatant was transferred to a silanized WISP insert, and 5 µl was injected onto the HPLC column.

By use of simple precipitation and centrifugation for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of fluorescence detection, halofantrine and WR 178,460 (as free bases) can be quantitatively and reliably measured in rat liver homogenate samples. The assay described in the report dated January, 28, 1997, requires 200 µl rat liver homogenate samples to determine the concentrations in the range 0.540 - 69.1 µg/ml halofantrine and WR 178,460 (as free bases). The method involves sample cleanup with acetonitrile precipitation, separation on a silica column run with an aqueous mobile phase, and fluorescence detection. The LLOQs for the assay of rat liver homogenate, based on interday and intraday low point validation results, have been set at 0.540 µg/ml for halofantrine and 0.540 µg/ml for WR 178,460 (as free bases). The overall mean recoveries over the working range of the methodology were 99.1% for halofantrine and 94.6% for WR 178,460 (as free bases). The result ranges for halofantrine interday were C.V. 2.52 to 5.45% and R.E. +0.514 to +5.62%. The ranges for halofantrine intraday were C.V. 2.33 to 4.86% and R.E. -0.685 to +10.4%. The ranges for WR 178,460 interday were C.V. 2.30 to 8.55% and R.E. -3.93 to +4.70%. The ranges for WR 178,460 intraday were C.V. 2.53 to 5.25% and R.E. -2.78 to +5.76%.

Study Report 18: WR 6026 and Metabolite in Human Plasma

Study Characteristics: Study Report 18

Test Article:	WR 6026, WR 211,789
Test System:	human plasma and blood
Internal Standard:	chlorpheniramine
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 263 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5μ particle size
Mobile Phase:	acetonitrile/water (3:2, v/v) final concentration of 5 mM $(NH_4)_2HPO_4$ at pH 8.8

Validation Results: WR 6026 in human plasma

Quantitation Limit:	0.980 ng/ml
Standard curve range:	0.980-98.0 ng/ml
Interday Precision	
Concentration Range:	2.06-77.3 ng/ml
CV Range:	3.05-6.82%
Intraday Precision	
Concentration Range:	2.06-77.3 ng/ml
CV Range:	3.22-9.39%
Blind Sample Assay	see Appendix A, Midterm Report
Mean Recovery:	74.5%
Stable Plasma Storage:	-20°C for 3 months

Validation Results: WR 211,789 in human plasma

Quantitation Limit:	1.21 ng/ml
Standard curve range:	1.21-121 ng/ml
Interday Precision	
Concentration Range:	2.14-80.1 ng/ml
CV Range:	5.19-8.98%
Intraday Precision	
Concentration Range:	2.14-80.1 ng/ml
CV Range:	4.42-7.86%
Blind Sample Assay	see Appendix A
Mean Recovery:	93.8%

Validation Results: WR 6026 in human blood

Quantitation Limit:	0.980 ng/ml
Standard curve range:	0.980-98.0 ng/ml
Interday Precision	
Concentration Range:	1.96-78.4 ng/ml
CV Range:	1.56-6.38%
Intraday Precision	
Concentration Range:	1.96-78.4 ng/ml
CV Range:	2.31-5.36%
Stable Plasma Storage:	-20°C for 1 month -70°C for 3 months

Validation Results: WR 211,789 in human blood

Quantitation Limit:	1.21 ng/ml
Standard curve range:	1.21-121 ng/ml
Interday Precision	
Concentration Range:	2.40-96.0 ng/ml
CV Range:	1.74-5.12%
Intraday Precision	
Concentration Range:	2.40-96.0 ng/ml
CV Range:	1.76-4.85%

Study Description: WR 6026 and Metabolite in Human Plasma

Sets of blind plasma and blood samples, prepared April 1, 1993, were received. Blind plasma sample results were enclosed with Quarterly Report 8. Upon analysis of blood samples, results will be forwarded to the COR. Acceptable results will be incorporated into Study Report 18, "Quantitation of WR 6026 and WR 211,789 (as Free Bases) in Plasma and Blood by High-Performance Liquid Chromatography." The test of stability is in progress.

Procedures Required to Complete Validation

The following list details changes that were instituted for plasma sample analysis, but that have not been tested for validation of the blood sample analytical method.

1. Following addition of 5 ml of methyl-*t*-butyl ether, vortex [not rotate] samples for 1 [not 15] min.
2. Adjust the mobile phase pH to 8.8 [not 7.0].
3. Stock and working solutions were stored at -20°C [not 4°C].

The following list details validation tests that have not been done.

1. Stability of WR 211,789 (free base) at -80°C and -20°C in blood and plasma.
2. Recovery of WR 6026 and WR 211,789 from blood.
3. Precision of WR 6026 and WR 211,789 (as free bases) in blood with mobile phase pH = 8.8, storage of stock and working solutions at -20°C, and vortexing extraction samples for 1 min.
4. Accuracy for WR 6026 and WR 211,789 (as free bases) in plasma and blood on blind spiked samples prepared by the Walter Reed Army Institute of Research.
5. Interference: To determine whether known compounds would interfere with detection of WR 6026 or WR 211,789 (as free bases), the retention times relative to CPA in mobile phase of several WR 6026 (free base) analogs could include WR 225,742 and WR 254,421 (free base).

Study Description

WR 6026 (dihydrochloride) (6-methoxy-8-(6-diethyl amino hexyl amino) lepidine dihydrochloride) (see figure below), is a very effective antileishmanial drug in hamsters infected with *Leishmania donovani* .[16]

Because antimony compounds are not always effective and the other drugs in use have toxic effects,[17],[18] alternative therapies are needed. Since WR 6026 (dihydrochloride) is a likely candidate and since WR 6026 (dihydrochloride) is scheduled for clinical testing in the near future, it is extremely important to develop an analytical method capable of measuring concentrations of WR 6026 (free base) at nanogram per milliliter concentrations in biological samples.

This report describes an assay developed to determine the concentrations of WR 6026 and of its mono dealkylated metabolite, WR 211,789, (as free bases) in blood and plasma. This new assay provides significant improvements over capabilities of earlier assays with increased sensitivity for the detection of WR 6026 (free base)[19]and inclusion of WR 211,789 (free base) in the methodology (Study Report 10).

Plasma samples (0.5 ml transferred with a plastic tipped pipetter to silanized culture tubes (see SOP #3-11 for silanization procedure)) were vortexed with 100 µl of a 1.00 µg/ml chlorpheniramine maleate internal standard working solution and 100 µl of a 1 N NaOH solution for 10 s. Next, 5 ml of methyl-*t*-butyl ether was added and samples were vortexed for 1 min, then centrifuged for 10 min at 3000 *g*. Then, for each sample, the aqueous layer was frozen in a dry ice/methanol bath and the organic layer were decanted into a new silanized culture tube. Finally, the sample's organic layer was evaporated to dryness under prepurified nitrogen, reconstituted in 200 µl of mobile phase, vortexed for 1 min, transferred to silanized WISP inserts, and injected onto the HPLC column.

Blood samples (0.5 ml transferred with a plastic tipped pipetter to silanized culture tubes) were vortexed for 1 min with 0.5 ml of nanopure water, and the mixtures were sonicated for 10 min. Then, these samples were prepared like plasma samples beginning with addition of 100 µl of the internal standard working solution.

No degradation of WR 6026 (free base) in plasma frozen at -20°C or blood frozen at -80°C was seen for the duration of the stability study. However, noticeable degradation of WR 6026 (free base) in blood frozen at -20°C was observed by the third month at all concentrations.

Two standard curves for each assay were constructed from the chromatographic data; a low range curve from the 0 to 14.7 ng/ml for WR 6026 and 0 to 18.1 ng/ml for WR 211,789 standard curve samples and a high range curve from the 0 to 98.0 ng/ml for WR 6026 and 0 to 121 ng/ml (i.e. all) standard curve samples in order to obtain more accurate determinations of low level WR 6026 and WR 211,789 (free base) concentrations. The low range standard curve was used to calculate drug or metabolite concentrations for assayed samples when the peak height ratio of the sample was less than or equal to the calculated peak height ratio at the highest concentration of the low range curve (as calculated from the low range curve). The high range curve was used to calculate results for samples with peak height ratios greater than the calculated peak height ratio at the highest concentration of the low range curve (as calculated from the low range curve).

Typical plasma and blood chromatograms show WR 6026 (free base), WR 211,789 (free base) and internal standard, chlorpheniramine, peaks that are baseline separated and separated from other components of the sample.

A linear relationships was demonstrated between the WR 6026 and WR 211,789 (free base) spiked concentrations to the WR 6026 and WR 211,789 (free base) to internal standard peak height ratios for the plasma and blood assays. Linear regression analysis of concentration versus the peak height ratio gave coefficients of determination (r^2) of 0.989 or better for these typical standard curves. The linear range of the standard curves covered WR 6026 (free base) concentrations in plasma and blood in the range 0.980 to 98.0 ng/ml and WR 211,789 (free base) concentrations in plasma and blood in the range 1.21 to 121 ng/ml. The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange[20] and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid

compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug.

Validation trials in our laboratory for an earlier study (Study Report 10) were undertaken to include in the WR 6026 (free base) assay the capability to measure WR 211,789 (free base), a mono dealkylated metabolite of WR 6026 (free base), concentrations in biological samples. Large variations between spiked and recovered concentrations were observed in that study. Although WR 211,789 has been detected in a rat microsomal preparation,[21] it has not been detected in plasma in human studies, perhaps because the detection limit of the assay used was only 10 ng/ml.[19] WR 211,789 plasma standard curves in the trials were of higher quality than blood standard curves. The current report describes an adaptation of the WR 6026 (free base) methodology or a modification of the methodology presented in the earlier report (Study Report 10), in which a 5 to 10 fold increase in sensitivity has been gained that makes detection of WR 211,789 (free base) in human plasma possible at higher WR 6026 (dihydrochloride) doses.

In addition, compared to an even earlier methodology,[19] the WR 6026 (free base) HPLC method presented here offers increased sensitivity and extends the range of biological fluids that can be assayed. The earlier method measured WR 6026 (free base) in plasma cleaned by protein precipitation (with acetonitrile) and column elution (from a C2 extraction column), had a 6.44 ng/ml WR 6026 (free base) detection limit, used WR 223,658 as an internal standard, required a C8 bonded silica gel HPLC column, used a 60:40 (v/v) acetonitrile/water mobile phase at pH 5.5 with 0.2% final concentrations of SDS and glacial acetic acid, and measured WR 211,789 (free base) with a minimum detection limit of 8 ng/ml. The newer method measures WR 6026 (free base) in plasma and blood cleaned by extraction with 99:1 (v/v) pentane/acetonitrile, has a 0.980 ng/ml WR 6026 (free base) detection limit, uses chlorpheniramine maleate as an internal standard, requires an unbonded silica gel HPLC column, uses a 70:30 (v/v) acetonitrile/water mobile phase at pH 7.0 with 5 mM final concentration of dibasic ammonium phosphate, but could not measure WR 211,789 (free base) with a minimum detection limit much better than 8 ng/ml. The current modified method measures WR 6026 and WR 211,789 (free base) in plasma and blood cleaned by extraction with methyl-*t*-butyl ether, has 0.980 ng/ml WR 6026 and 1.21 ng/ml WR 211,789 (as free bases) detection limits, uses chlorpheniramine maleate as an internal standard, requires an unbonded silica gel HPLC column, uses a 60:40 (v/v) acetonitrile/water mobile phase at pH 8.8 with 5 mM final concentration of dibasic ammonium phosphate.

HPLC assays for basic amine drugs in biological samples that make use of a silica gel column and an aqueous mobile phase have been operated in this

laboratory for over 5 years.[22,23,24] In the WR 6026 (free base) HPLC method presented here, the use of an unbonded silica gel column, an aqueous mobile phase, and UV detection at 263 nm yields satisfactory results for the determination of WR 6026 and WR 211,789 (as free bases) in (0.5 ml) plasma and blood samples. The method is simple in that a single extraction step and evaporation of solvent prior to injection are required. Recovery of WR 6026 (free base) averaged 74.5%, while recovery of WR 211,789 (free base) averaged 93.8% from plasma. The minimum quantitation limits of the assay were 0.980 ng/ml for WR 6026 (free base) and 1.21 ng/ml for WR 211,789 (free base) for blood and plasma. The coefficients of variation of the inter- and intraday assay precision analyses were less than 10% at all concentrations. The method is simple, precise, more sensitive, and includes the capability of quantitating WR 211,789 (free base) as well as the parent drug compared to earlier methods.

Study Report 19: Mefloquine in Human Blood

Study Characteristics: Study Report 19

Test Article:	Mefloquine
Test System:	human blood
Internal Standard:	chlorpheniramine
Sample Assay Volume:	0.5 ml
Sample Cleanup:	pentane/methylene chloride (7:3, v/v) extraction

Analytical System

Detector:	UV at 280 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	methanol/water (4:1, v/v) final concentration of 5 mM $(NH_4)_2HPO_4$ at pH 7.5

Validation Results: Mefloquine in blood

Quantitation Limit:	7.36 ng/ml
Standard curve range:	7.36-2210 ng/ml
Interday Precision	
Concentration Range:	14.7-1472 ng/ml
CV Range:	3.94-8.41%
Intraday Precision	
Concentration Range:	14.7-1472 ng/ml
CV Range:	2.74-10.9%
Blind Sample Assay	
Concentration Range:	11.52-1536 ng/ml
Bias Range:	-12.6 to +7.20%
Mean Recovery:	91.5%
Stable Blood Storage:	-20°C for 4 months

Study Description: Mefloquine in Human Blood

Mefloquine (hydrochloride), (WR 142,490: erythro-a-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol hydrochloride), is an alternative curative agent for the treatment of falciparum malaria.[25,26] Mefloquine (hydrochloride) has also been shown to prophylactically suppress mosquito induced infections by *Plasmodium vivax* and *P. falciparum* in human volunteers.[27,28] Published plasma and/or blood analytical methods employ gas-liquid chromatography (GLC)[29,30,31] thin layer chromatography (TLC),[32]

gas chromatography-mass spectrometry (GC-MS)[33] or high performance liquid chromatography (HPLC) (Study Reports 6 and 14).[34,3536,37 38,39,40] The GLC methods require derivitization, and sample volume in the method described by Nakagawa, *et al.* uses 5 ml samples. The TLC method has no internal standard and is insufficiently sensitive. The GC-MS method requires derivitization and the increased expense of mass spectrometry.

Many HPLC have been reported. The method reported by Grindel, *et al.*, required three times extraction from 5 ml plasma samples and, upon solvent evaporation, the residues required overnight storage in a vacuum desiccator. Kapetanovic, *et al.* used a 3 step extraction of 1 ml samples. Our earlier study (Study Report 6) described a protein precipitation method for 0.2 ml plasma samples. Franssen, *et al.*, described a method for plasma and blood analysis for mefloquine and its carboxylic acid metabolite with 50 ng/ml mefloquine and 100 ng/ml metabolite detection limits. Karbwang, *et al.*, described a 50 ng/ml detection limit, 100 ng/ml quantitation limit method for mefloquine in blood and plasma. Coleman, *et al.*, measured mefloquine at 10 ng/ml in liver perfusate. Riviere *et al.*, presented a method with a 20 ng/ml detection limit and 100 ng/ml quantitation limit in plasma. Bergqvist, *et al.*, describe two HPLC methods for determination of mefloquine and its principal metabolite in plasma and blood, the first with 30 ng/ml plasma and 150 ng/ml blood quantitation limits and the second with 75 ng/ml quantitation limits for both compounds.

We reported (Study Report 14) the development of a simple and rapid HPLC assay for mefloquine (free base) that requires 0.5 ml plasma samples and a one step extraction, has an 7.36 ng/ml quantitation limit and produces chromatograms with a cleaner baseline than our previous method. Study Report 19 describes the extension of our plasma method to include analysis of blood samples. Study Report 19 also describes status of steps taken toward extension of the method for determination of the main mefloquine metabolite, WR 160,972 (2,8-bis-(trifluoromethyl)-4-quinoline carboxylic acid).[41]

The blood method 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.

Blood samples for analysis are pipetted (0.5 ml) into screw top tubes. Add 100 µl of a saturated solution of sodium carbonate and vortex the mixture for

1 min. Then, add 100 µl of the internal standard working solution (CPA, 12 µg/ml) and vortex the mixture for 1 min. The sample is extracted with 5 ml of pentane/methylene chloride (7:3, v/v), evaporated to dryness under nitrogen, resuspended in 200 µl of mobile phase and injected (40-80 µl) onto the HPLC column.

An addendum with blind sample results enclosed with Quarterly Report 6 completed Study Report 19 (Status Report, dated January 14, 1992 and titled "Quantitation of Mefloquine (Free Base) in Blood by High-Performance Liquid Chromatography, Extraction Method." Further work on this assay is scheduled to include WR 160,972 method development, but work on this aspect of the project has been assigned a low priority by the COR.

Study Report 20: Artelinic Acid in Human Plasma

Study Characteristics: Study Report 20

Test Article:	artelinic acid (WR 255,663)
Test System:	human plasma
Internal Standard:	meclofenamic acid
Sample Assay Volume:	1 ml
Sample Cleanup:	Precipitation with acetonitrile SAX ion exchange solid phase extraction

Analytical System

Detector:	UV at 236 nm
Column Type:	C18 bonded silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	acetonitrile/water (1:1, v/v) 50 mM $NH_4H_2PO_4$ at pH 5.00.

Validation Results: artelinic acid

Quantitation Limit:	4.96 ng/ml
Interday CV:	19.8%
Interday Error:	8.47%
Standard curve range:	4.96-1270 ng/ml
Interday Precision	
Concentration Range:	9.96-797 ng/ml
CV Range:	2.62-8.97%
Intraday Precision	
Concentration Range:	9.96-797 ng/ml
CV Range:	1.76-7.81%
Blind Sample Assay	
Concentration Range:	23.54-401.4 ng/ml
Bias Range:	-13.1 to +0.52%
Mean Recovery:	85.3%
Stable Plasma Storage:	-20°C for 6 months

Study Description: Artelinic Acid in Human Plasma

Malaria has continued to be a major health problem in many regions of the world for two reasons. One is that the Anopheles mosquito has developed resistance towards a number of insecticides widely used for vector control. But, the more alarming reason is that the malarial parasite has

developed resistance to chloroquine and mefloquine. This phenomenon is rapidly increasing in both degree and prevalence throughout the world.[42,43] As a result, the search for more effective antimalarial agents with differing mechanisms of action is more important than ever before. There is considerable interest in developing natural products as antimalarials, and one of the prime candidates to be examined is qinghaosu (QHS), also known as artemisinin (I, Fig. 1). This compound was isolated from the plant *Artemisia annua* L. and characterized by the Chinese in 1972.[44,45,46] The herb itself has been known to Chinese medicine since 160 BC.[46] Qinghaosu, which has been used effectively for the treatment of malaria, is a sesquiterpene lactone with an endo-peroxide bridge. A number of derivatives of the parent compound have been prepared, among which the methyl ether of reduced QHS, called artemether (II, Fig. 1), dihydroqinghaosu (DQHS) (III, Fig. 1) and the succinate hemi-ester of DQHS, artesunic acid (IV, Fig. 1) and its sodium salt are currently used in China for the treatment of multi-drug resistant malaria patients with excellent results.[47] The Steering Committee of the Scientific Working Group on Malaria Chemotherapy of the World Health Organization in Geneva, Switzerland (SWG-CHEMAL), selected the ethyl ether of DQHS, β–arteether (V, Fig. 1), for clinical investigation.[48]

Qinghaosu and its derivatives were found to be thermally labile[49,50,51] and lack UV chromophores or functional groups for derivative formation. Sensitive and selective analytical methods are essential for studying metabolic and pharmacological aspects of drugs and development of such methods for these new drugs has been challenging chemists for some time. Among the several methods developed for the estimation of qinghaosu and its derivatives mentioned previously are included a radioactivity measure of tritium labeled drug following TLC separation, a GC-MS method, HPLC with UV detection after derivatization or degradation and HPLC separation with direct reductive electrochemical detection. Two different modes of HPLC determination of these drugs in biological fluids have been attempted; UV detection of a derivative or pre-column acid or base-catalyzed decomposition product which is UV absorbing after HPLC separation,[52,53,54,55] and reductive electrochemical detection using gold/mercury amalgam or glassy carbon electrodes following HPLC separation.[48,56] The first mode lacks specificity in that metabolites of the drugs are also liable to be converted to identical UV absorbing products. Furthermore, the derivative formation can take place only with compounds having a suitable functional group. For example, DQHS with an OH group at position 12 can react with diacetyl dihydro fluoresceein to form an intensely UV absorbing derivative.[57] This method can detect DQHS at concentrations as low as 18 ng/ml, with good reproducibility, and the limit of detection is given as 0.1 ng of DQHS at 235 nm UV wavelength. The chemical structure of β–arteether contains no such functional group, thus eliminating derivatization as a method for increasing UV detectability. It is reported that base catalyzed degradation is effective only

for QHS and artesunic acid but not for β–arteether. The acid catalyzed decomposition of β–arteether followed by UV detection has been reported and validation of the assay down to 60 ng/ml of plasma was provided. Bearing in mind the previously mentioned shortcomings of pre-column degradative methods, the reported assay method for β–arteether cannot be considered completely satisfactory. The reductive electrochemical detection following HPLC separation (LC-EC) is the only highly sensitive (5 ng/ml) method available presently for the determination of β–arteether in plasma. Since the detector specifically reduces the peroxide bridge in the compound, interference from many other compounds extracted from plasma is eliminated. Thus, the technique is both sensitive and selective for these drugs. However, the reductive electrochemical detector has several practical difficulties inherent to it which makes its use as a routine analytical method difficult. Free oxygen is easily reduced at the detector electrode and this gives rise to a large signal. Accordingly, the following precautions have to be rigorously adhered to in order to obtain the expected results: 1. the complete HPLC and detector system need purging with inert gas to eliminate aerial oxygen; 2. the mobile phase has to be continuously purged to keep the system oxygen free; and 3. the samples have to be injected through an oxygen free system, such as a specially designed autosampler which automatically deoxygenates and injects samples.

It was felt that a method for the estimation of β–arteether in plasma, which was less demanding in instrumental facilities than reductive electrochemical detection following HPLC would be of value for routine laboratory analysis of large numbers of samples. Attempts to develop such an assay here are described in the discussion. Studies on three types of possible detection systems following HPLC separation are reported: i. oxidative electrochemical detection; ii. UV detection both following post column photochemical activation; and iii. a chemical derivatization followed by UV detection, since the two previous methods were not successful. An analytical procedure using the third detection system was not capable of quantitating β–arteether with a minimum quantitation limit greater than currently available methods.

In view of the outcome of these studies on the detection of β–arteether in plasma, project emphasis for this laboratory was shifted towards development of a methodology for quantitation of artelinic acid (VI, Fig. 1), which is synthetically derived from quinghaosu, has a water soluble sodium salt, is stable in potassium carbonate solution, has demonstrated antimalarial activity against *Plasmodium berghei* in mice, and is considered a candidate drug for cerebral malarial treatment.[58] An HPLC assay for artelinic acid in plasma with an estimated 50 ng/ml detection limit was reported by Idowu, et al.[59] We present an approach to the HPLC analysis of a plasma sample for determination of the concentration of artelinic acid that employs a solid

phase extraction step with a trimethyl aminopropyl or an aminopropyl bonded elution cartridge. Chromatographic separation was accomplished with an ODS column run with an acetonitrile/50 mM $NH_4H_2PO_4$ (1:1, v/v), pH adjusted to 5.00 with H_3PO_4 mobile phase. UV absorbance was measured at 236 nm. The system produced chromatograms with clean baselines and high signal to noise ratios at low concentrations. The minimum quantitation limit of the method for plasma samples is approximately 5 ng/ml.

The assay requires that all plasma samples must be stored at a temperature no higher than -20°C.

Plasma samples for analysis are pipetted (1 ml) into culture tubes and vortexed. A constant amount, approximately 160 ng, of the internal standard, meclofenamic acid, was added. The samples are vortexed, precipitated with 2 ml acetonitrile, then evaporated to 200 µl and combined with 1 ml water. The aqueous solution is loaded onto a 500 mg SAX (or NH2) elution cartridge (prepared by sequential washes with 3 ml methanol , 2 ml (6 ml for NH2 cartridge) 0.1 M hydrochloric acid, 3 ml water and 6 ml 0.1 M monobasic ammonium phosphate). The cartridges are then washed with 3 ml water, 3 ml acetonitrile (completely vacuum dry NH2 cartridge), and 0.5 ml 0.5 M formic acid (wash NH2 cartridge twice with formic acid and vacuum dry) in acetonitrile. The compounds are eluted from the cartridges with 2 ml 0.5 M formic acid in acetonitrile (use three 0.5 ml aliquots of formic acid for the NH2 cartridge). The eluents are evaporated to dryness under N_2 and reconstituted in 200 µl of 50% acetonitrile. Then, 60 µl (30 µl for the NH2 cartridge) is injected onto the HPLC column.

All validation tests were performed with the NH2 cartridge elution sample clean up procedure. For comparison, the SAX cartridge elution sample clean up procedure was used to repeat the precision and recovery tests.

An ODS column and a mobile phase composition of acetonitrile/50 mM monobasic ammonium phosphate (1:1, *v/v*) with pH adjusted to 5.00 with phosphoric acid were used to separate artelinic acid from the internal standard and interfering endogenous substances in an isocratic elution.

The minimum quantitation limit (MQL), approximately 5 ng/ml, was determined as the artelinic acid concentration at which the signal to noise ratio was at least 3 to 1. Table 2 compares the NH2 and SAX method MQL's, at the lowest and second to the lowest points of the standard curve.

Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level drug concentrations, two standard curves were constructed from the same set of standard curve data points. Data points from 0 to 79.3 ng/ml were used to construct a low artelinic acid concentration standard curve with which to calculate the low concentration

samples. All data points (0 to 1270 ng/ml) were used to construct a high artelinic acid concentration standard curve for the high concentration samples. Concentrations of samples with peak height ratios above that calculated at 79.3 ng/ml from the low concentration standard curve were calculated with the high concentration standard curve.

Typical SAX method chromatograms for blank plasma samples and plasma samples spiked with artelinic acid and the internal standard, meclofenamic acid show early elution of most endogenous interferences and baseline separation of the two compounds, with retention times of artelinic acid at approximately 19 minutes and the internal standard at approximately 16 minutes.

For the representative SAX method standard curve, the coefficients of determination for artelinic acid in plasma were 0.9989 and 0.9994 for low and high concentration ranges, respectively. Percent errors for the interday mean calculated concentration of replicate analyses of samples spiked at the lowest point of the standard curve (the minimum quantitation limit) were 7.45% for the NH2 method (n=5) and 8.47% for the SAX method (n=4).

Meclofenamic acid was found to be an acceptable internal standard since amounts used were below quenching levels, the retention time was sufficiently later than early eluting interferences, and the retention time was sufficiently close to that of artelinic acid to limit error caused by changes in peak shape that arise as columns age with use.

We tested coumarin derivatization with an earlier sample preparation procedure[60] to enable fluorescence detection. Since many peaks were detected, coumarin is postulated to react with hydroxy, amino, and/or carboxylic moieties. A gradient elution program was required to separate late peaks from artelinic acid (Figure 3). Ibuprofen was also derivatized and used as the internal standard, but produced a double peak (not shown). The detection limit was estimated to be comparable with that of the UV method.

WRAIR has GS-MS and HPLC-EC reductive mode methods available to perform routine analysis of plasma samples for determination of the β–arteether concentrations. Our initial investigations did not reveal promising avenues for an improved β–arteether method. Thus, our efforts towards developing improved methods for quantitation of qinghaosu derivatives were shifted by WRAIR from continued study of a β–arteether method to a search for an improved artelinic acid method.

Since the fluorescence method required derivatization and gradient elution to achieve about the same sensitivity as the UV method, it was judged a currently unacceptable method to proceed with validation. A

dicarboxylic acid derivative is being considered as an internal standard for future fluorescence detection experiments.

We observed and evaluated a lot dependent inconsistency with NH_2, 500 µg ion-exchange cartridges during our validation of stability for this methodology. A quaternary aminopropyl elution cartridge (SAX ion exchange) was tested that eliminated the lot dependent inconsistency and that was shown to have comparable precision and improved recovery relative to the NH_2 ion-exchange cartridge. The recovery, precision and blind sample assays were, and any impending studies should be, performed with the SAX cartridge sample clean up procedure.

By use of a solid phase extraction step for sample clean-up, an ODS column combined with an acetonitrile/ 50 mM $NH_4H_2PO_4$ (1:1, v/v), pH adjusted to 5.00 with H_3PO_4 mobile phase for separation, and the superior capability of ultraviolet detection, artelinic acid can be quantitatively and reliably measured in biological samples. The method is improved over our earlier method by a 4 fold increase in sensitivity and elimination of the use of silanized glassware.[60] The assay described in the report dated July, 27, 1994, requires 1 ml plasma samples to determine the concentrations of artelinic acid. The method involves sample cleanup with quarternary amine solid phase extraction cartridge columns, separation on an ODS column run with an aqueous mobile phase, and ultraviolet detection. The minimum artelinic acid quantitation limit for the assay of plasma is approximately 5 ng/ml with a signal to noise ratio of 3 to 1. Metabolites of artelinic acid should derive from liberation of benzoic acid and should retain chromophores which may induce chromatographic interferences, but would probably be sufficiently polar to elute sufficiently early to be non-interfering in the assay. Mean recoveries over the working range of the methodology ranged from 80.1% to 88.5% for SAX cartridges. The SAX method coefficients of variation (CVs) of the inter- and intraday assay precision analysis for the concentrations of artelinic acid ranged from 1.76 to 7.81% for intraday precision and 2.62 to 8.97% for interday precision, respectively, in plasma. Blind sample assay percent bias ranged -13.1 to +0.523% when SAX cartridge elution is used. Drug stability in human plasma was demonstrated for storage up to 6 months at -20°C and for repeated sample analysis.

Validation Study Report 20C, dated July 27, 1994 and titled "Quantitation of Artelinic Acid in Human Plasma by High Performance Liquid Chromatography with a C18 Bonded Column," was submitted as final with the COR's approval, dated May 13, 1994, and appended to quarterly report 10. Complete validation results are presented in Appendix A

Study Report 21: *p*-Aminoheptanophenone and Metabolites in Dog Plasma and Rat Plasma

Study Characteristics: Study Report 21

Test Article:	WR 269,410 (*p*-aminoheptanophenone)
Test System:	dog plasma
Internal Standard:	WR 258,948 (*p*-aminooctanophenone)
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 316 nm
Column Type:	C18 bonded silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	acetonitrile/water (1:1, v/v) and 0.15% H_3PO_4

Validation Results:

Quantitation Limit:	4.08 ng/ml
Standard curve range:	4.08-816 ng/ml
Blind Sample Assay	see Appendix A

Study Description: *p*-Aminoheptanophenone and Metabolites in Dog Plasma and Rat Plasma

Method validation will be reported in Study Report No. 21 (preparation in progress covering *p*-aminoheptanophenone (PAHP, WR 269,410) *p*-aminooctanophenone (PAOP, WR 258,948) and *p*-aminopropiophenone (PAPP, WR 302)). Results from the analysis of blind spiked (by WRAIR) dog plasma samples were presented in Appendix A of the mid-term report.

Study Report 22: WR 6026 and Metabolites in Human Urine

Study Characteristics: Study Report 22

Test Article:	WR 6026 WR 211,789 WR 254,421
Test System:	human urine
Internal Standard:	verapamil
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	acetonitrile/0.0075% phosphoric acid (80:20, v/v) at pH 6.9.

Validation Results WR 6026 free base

Quantitation Limit:	5.17 ng/ml
Interday CV:	14.8%
Interday Error:	2.44%
Standard curve range:	2.17-414 ng/ml
Interday Precision	
Concentration Range:	10.4-259 ng/ml
CV Range:	3.90-7.42%
Intraday Precision	
Concentration Range:	10.4-259 ng/ml
CV Range:	3.83-28.4%
Blind Sample Assay	
Concentration Range:	5.20-101.2 ng/ml
Bias Range:	-10.6 to +33.9%
Mean Recovery:	97.2%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Validation Results WR 211,789 free base

Quantitation Limit:	509 ng/ml
Interday CV:	14.7%
Interday Error:	4.55%
Standard curve range:	5.09-407 ng/ml
Interday Precision	
Concentration Range:	10.2-255 ng/ml
CV Range:	4.07-10.0%
Intraday Precision	
Concentration Range:	10.2-255 ng/ml
CV Range:	5.12-23.3%
Blind Sample Assay	
Concentration Range:	5.20-102.6 ng/ml
Bias Range:	-11.8 to +33.9%
Mean Recovery:	92.8%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Validation Results WR 254,421 free base

Quantitation Limit:	45.4 ng/ml
Interday CV:	7.51%
Interday Error:	1.60%
Standard curve range:	45.4-3630 ng/ml
Interday Precision	
Concentration Range:	90.8-2270 ng/ml
CV Range:	3.09-5.86%
Intraday Precision	
Concentration Range:	90.8-2270 ng/ml
CV Range:	3.55-10.0%
Blind Sample Assay	
Concentration Range:	50.1-979.4 ng/ml
Bias Range:	-10.7 to +9.63%
Mean Recovery:	94.2%
Stable Plasma Storage:	-70°C for 4 months
Stable Prepared Sample:	Room temp. for 48 hours

Study Description: WR 6026 and Metabolites in Human Urine

Study Report No. 22 "Quantitation of WR 6026, WR 211,789 and WR 254,421 (as Free Bases) in Human Urine by High Performance Liquid

Chromatography," was submitted for review July 18, 1994. This method is a modified version of the plasma method.

WR 6026 (dihydrochloride) (6-methoxy-8-(6-diethyl amino hexyl amino) lepidine dihydrochloride) (Figure 1), is a very effective antileishmanial drug in hamsters infected with *Leishmania donovani* .[16] Because antimony compounds are not always effective and the other drugs in use have toxic effects,[17,18] alternative therapies are needed. Since WR 6026 (dihydrochloride) is a likely candidate and since WR 6026 (dihydro-chloride) is scheduled for clinical testing, it is extremely important to develop an analytical method capable of measuring concentrations of WR 6026 (free base) at nanogram per milliliter concentrations in biological samples. This report describes an assay developed to determine the concentrations (as free bases) of WR 6026 and of its metabolites, WR 211,789 (6-methoxy-8-(6-ethyl-aminohexylamino) lepidine dihydrochloride, hemihydrate) and WR 254,421 (8-(6'-N,N-diethylaminohexyl)amino-4-hydroxymethyl-6-methoxyquinoline, dihydrochloride) in urine. WR 211,789 has been detected in a rat microsomal preparation.[21] This assay adds the capability of detection of WR 6026, WR 211,789 and WR 254,421 (as free bases) in urine to earlier assays for WR 6026 and WR 211,789 in plasma and blood.

Assay samples were prepared by spiking known volumes of human urine with a known amount (constant over all samples) of the verapamil internal standard (IS). Standard curve samples were generated by spiking interference free human urine samples with known amounts of WR 6026, WR 211,789 and WR 254,421 (as free bases) and IS. These standard curve and assay samples were extracted, then injected onto an HPLC column for separation and subsequent ultraviolet detection. The peak height ratios of WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Finally, standard curve concentrations and WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS peak height ratios of the standard curve samples were fit by least squares linear regression to the equation for the best straight line ($y = mx + b$, where y = peak height ratio and x = WR 6026, WR 211,789 or WR 254,421 (as free bases) concentrations), and drug concentrations in assay samples were calculated by this equation from the WR 6026, WR 211,789 and WR 254,421 (as free bases) to IS peak height ratios obtained by HPLC.

Sample volume taken for analysis was 0.5 ml of urine. A constant amount, approximately 5 µg, of the internal standard, verapamil, was added to and mixed with each sample. Next, 100 µl of 1 N NaOH was added to and mixed with each sample. Then, samples were extracted with 5 ml of methyl *t*-butyl ether. The extraction solution was transferred to a second culture tube, evaporated to dryness under nitrogen, and reconstituted in 200 µl of mobile phase. Finally 20-160 µl of the sample was injected onto the HPLC column.

In typical chromatograms for blank urine and urine samples spiked with WR 6026, WR 211,789 or WR 254,421, WR 6026, WR 211,789 or WR 254,421 eluted at 15.3, 14.3, and 18.2 minutes, respectively, and the internal standard eluted at 12.4 minutes. The coefficients of determination for WR 6026, WR 211,789 or WR 254,421 interday and intraday precision standard curves were 0.9825 or higher.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug.

HPLC assays for basic amine drugs in biological samples that make use of a silica gel column and an aqueous mobile phase have been operated in this laboratory for over 5 years. By use of an organic solvent extraction step for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of ultraviolet detection, the free base concentrations of WR 6026, WR 211,789 and WR 254,421 can be quantitatively and reliably measured in human urine samples. The assay described in the report dated July, 18, 1994, requires 0.5 ml urine samples to determine the free base concentrations of WR 6026, WR 211,789 or WR 254,421. The method involves sample cleanup with a methyl *t*-butyl ether extraction, separation on an unbonded silica gel column (5 µm particle size) run with an aqueous mobile phase, and ultraviolet detection. The minimum quantitation limits of the assay are 5.17, 5.09, and 45.4 ng/ml for WR 6026, WR 211,789 and WR 254,421 free base, respectively, with a signal to noise ratio of 3 to 1. Average mean recoveries over the working range of the standard curve were 97.2, 92.8, and 94.2 percent for WR 6026, WR 211,789 and WR 254,421 free base, respectively. The respective percent coefficients of variation (CVs) of the inter- and intraday assay precision analysis for the free base concentrations of WR 6026 ranged from 3.90% to 7.42% and 3.83% to 28.4%; of WR 211,789 ranged from 4.07% to 10.0% and 5.12% to 23.3%; and of WR 254,421 ranged from 3.09% to 5.86% and 3.55% to 10.0%. No discernible pattern of degradation was observed in long term or autosampler stability tests.

Study Report 23: Primaquine and Metabolite in Human Plasma

Study Characteristics: Study Report 23

Test Article:	primaquine phosphate (WR 002,975), carboxy-primaquine (WR 249,725)
Test System:	human plasma
Internal Standard:	mebendazole
Sample Assay Volume:	0.5 ml
Sample Cleanup:	double ether acetate extraction

Analytical System

Detector:	UV at 280 nm
Column Type:	C18 bonded silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	methanol/acetonitrile/water/phosphoric acid/TMACl (33%, 18.7%, 48%, 0.13%, 0.2%) with a final pH of 5.05.

Validation Results: primaquine free base in human plasma

Lower Limit of Quantitation:	28.5 ng/ml
Interday Mean, CV and RE:	33.8 ng/ml, 4.60% and +18.7%
Intraday Mean, CV and RE:	33.9 ng/ml, 6.04% and +18.8%
Standard curve range:	28.5 to 2850 ng/ml
Interday Precision Concentrations:	85.4, 171, 456, and 1420 ng/ml
CV Range:	2.34 to 3.58%
RE Range:	-5.02 to -1.37%
Intraday Precision Concentrations:	85.4, 171, 456, and 1420 ng/ml
CV Range:	1.34 to 3.68%
RE Range:	-3.51 to +1.06%
Blind Sample Assay	Not Run
Overall Mean Recovery:	101%
Stability	
Plasma Freezer Storage:	-70°C for 6 months -20°C for 3 months
Processed Sample:	10°C for 48 hours
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	6 months, protected from light

Validation Results: WR 249,725 free base in human plasma

Lower Limit of Quantitation:	20 ng/ml
Interday Mean, CV and RE:	19.6 ng/ml, 16.3% and -2.00%
Intraday Mean, CV and RE:	18.1 ng/ml, 8.01% and -9.70%
Standard curve range:	20.0 to 5000 ng/ml
Interday Precision Concentrations:	150, 300, 800, and 2500 ng/ml
CV Range:	3.19 to 4.73%
RE Range:	-0.278 to +1.61%
Intraday Precision Concentrations:	150, 300, 800, and 2500 ng/ml
CV Range:	1.23 to 7.91%
RE Range:	-1.20 to +8.00%
Blind Sample Assay	Not Run
Overall Mean Recovery:	75.0%
Stability	
Plasma Freezer Storage:	-70°C for 6 months
	-20°C for 3 months
Processed Sample:	10°C for 48 hours
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -20°C
Standard Solution:	6 months, protected from light

Study Description: Primaquine and Metabolite in Human Plasma

Primaquine has long ago been shown to protect against vivax and falciparum malaria.[61,62] This report describes validation of an adaptation of the assay method described by Baker, *et. al.* [63]for the determination of primaquine (free base) and its carboxylated metabolite concentrations in human plasma.

The plasma samples were analyzed for free base concentrations of WR 002975 (primaquine diphosphate) [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline] and for WR 249725 (carboxylated metabolite) [8-(3-carboxy-1-methylpropyl-amino)-6-methoxyquinoline], with an HPLC procedure that uses a C18 bonded column, an (methanol/acetonitrile/water) aqueous mobile phase, an ultraviolet detector, and 0.5 ml human plasma samples. Sample cleanup consisted of a double extraction into ethyl acetate, evaporation of the organic phase and reconstitution of plasma sample extracts in acetonitrile/water prior to separation by HPLC. The attached method SOP (Appendix A) contains detailed procedures and results, which are summarized below.

Assay samples were prepared by spiking known volumes of human plasma with a known amount (constant over all samples) of mebendazole IS. Standard curve samples were generated by spiking interference free human plasma samples with known amounts of primaquine (free base), its

carboxylated metabolite and IS. These standard curve and assay samples were extracted, then 10 to 25 µl aliquots were injected onto the HPLC column for chromatographic separation and subsequent ultraviolet detection. The peak height ratios of primaquine (free base) and its carboxylated metabolite to IS were calculated for each sample from the measured peak heights obtained by HPLC. Next, standard curve concentrations and primaquine (free base) and its carboxylated metabolite to IS peak height ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the two equations for the best straight lines ($y = mx + b$, where y = peak height ratio and x = primaquine (free base) or its carboxylated metabolite concentrations). Finally, drug and metabolite concentrations in assay samples were calculated by these equations from the primaquine (free base) and its carboxylated metabolite to IS peak height ratios obtained by HPLC.

Human plasma samples to be assayed for primaquine (free base) and the carboxylated metabolite are thawed at room temperature, then 0.5 ml of each sample is transferred to glass culture tubes and 100 µl of IS (mebendazole, 1.5 µg/ml) solution is added and mixed by vortexing for 10 s. Then, 3 ml of ethyl acetate extracting solvent is added, the mixture is vortexed twice for 30 s and the sample is centrifuged for 10 min at 3000 *g*. For each sample, the aqueous layer is frozen in a dry ice/methanol bath and the organic layer is transferred to a clean glass tube and evaporated to dryness under nitrogen. Then, the extraction of the aqueous layer is repeated with 2 ml of ethyl acetate and the organic layer is again evaporated to dryness. The residue is reconstituted in 100 µl of acetonitrile/water (2:3, v/v). The sample is vortexed 1 min, centrifuged at 3000 *g* 10 to 15 min, transferred to a WISP insert, and injected onto the HPLC column.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed.

By use of a double extraction with ethyl acetate for sample clean-up, a C18 bonded silica gel column combined with an aqueous mobile phase for separation, and ultraviolet absorption detection, the concentrations of primaquine (free base) and its carboxylated metabolite can be quantitatively and reliably measured in 0.5 ml human plasma samples. The LLOQs of the assay are 28.5 ng/ml for primaquine (free base) and 20.0 ng/ml for the carboxylated metabolite with signal to noise ratios of at least 3 to 1. The overall average recoveries (of averages taken at each of 4 concentrations) over the working range of the standard curves were 101% for primaquine (free base) and 75.0% for its carboxylated metabolite. The respective percent CVs of the inter- and intraday assay precision analysis for the free base concentration

of primaquine ranged from 2.34 to 3.58% and 1.34 to 3.68%. The respective percent CVs of the inter- and intraday assay precision analysis for the concentration of its carboxylated metabolite ranged from 3.19 to 4.73% and 1.23 to 7.91%. No discernible pattern of degradation was observed in long term (3 months at -20°C and 6 months at -70°C), bench top (6 hours at room temperature), system (48 hours at ≤10°C) or five freeze (at -20°C) thaw (at room temperature) cycle stability tests.

Study Report 24: Gentamicin and Paromomycin in Human and Rat Plasma

Study Characteristics: Study Report 24

Test Article:	Gentamicin Paromomycin
Test System:	human plasma rat plasma
Internal Standard:	Sisomicin
Sample Assay Volume:	0.2 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System: HPLC

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	16% CH_3CN, 0.2 M Na_2SO_4, 0.02 M sodium octanesulfonate, 0.1% acetic acid

Validation Results: Gentamicin free base in human plasma

Lower Limit of Quantitation:	0.100 µg/ml
Interday Mean, CV and RE:	7.88% and 7.00%
Intraday Mean, CV and RE:	15.7% and -14.9%
Standard curve range:	0.100-12.0 µg/ml
Interday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	4.34-8.42%
RE Range:	-3.20 to -0.800%
Intraday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	2.30-3.40%
RE Range:	-7.00 to -1.00%
Blind Sample Assay	
Concentration Range:	0.200-8.00 µg/ml
Bias Range:	-25.9 to +0.5%
Mean Recovery:	93.4%
Stability	
Plasma Freezer Storage:	-70°C for 12 months -20°C for 1 month
Processed Sample:	Room temp. for 3 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Validation Results: Paromomycin free base in human plasma

Lower Limit of Quantitation:	0.100 µg/ml
Interday Mean, CV and RE:	0.0960 µg/ml, 11.3% and -4.00%
Intraday Mean, CV and RE:	0.0867 µg/ml, 9.92% and -13.3%
Standard curve range:	0.100 to 12.0 µg/ml
Interday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	5.86 to 9.00%
RE Range:	-4.50 to +2.00%
Intraday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	3.12 to 3.96%
RE Range:	-7.50 to +1.87%
Blind Sample Assay	
Concentration Range:	0.200-9.00 µg/ml
RE Range:	-3.27 to +2.5%
Overall Mean Recovery:	93.8%
Stability	
Plasma Freezer Storage:	-70°C for 12 months
	-20°C for 1 month
Processed Sample:	Room temp. for 3 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Study Description: Gentamicin and Paromomycin in Human Plasma

This report describes a high performance liquid chromatographic (HPLC) assay and provides data validating the assay for the aminoglycosides gentamicin and paromomycin. These compounds are used in a topical preparation for the treatment of cutaneous leishmaniasis.

Assays for gentamicin described in the literature include HPLC/MS/MS in bovine kidney,[64] fluorescent polarization immunoassay (FPIA) in dog and rabbit tears,[65] and a dipstick dot-ELISA in dairy milk.[66] Also, recently described is a comparative evaluation of FPIA and an automated homogeneous latex agglutination immunoassay for gentamicin.[67] Recent antimicrobial assays for paromomycin have been described for anticryptosporidial activity[68] and respiratory burst response of *Leishmania major* infected murine macrophages.[69] Standard anti-leishmanial drugs may be tested for ability to inhibit growth of intracellular amastigotes of *L. aethiopica, L. donovani* and *L. infantum* with use of the human leukemia monocyte cell line, THP-1.[70]

This report presents validation data for a method that employs an aqueous mobile phase, a C18 column, post column derivitization and fluorescence

detection for concentration determinations in 0.2 ml human plasma samples with lower limits of quantitation of 0.100 µg/ml for both paromomycin and gentamicin.

Plasma samples were analyzed for gentamicin and paromomycin with an HPLC procedure that uses a C18 bonded column, an (acetonitrile/water) aqueous mobile phase, a *o*-pthaldialdehyde and 2 mercaptoethanol post column reagent, a fluorescence detector, and a 0.2 ml method sample size. Sample cleanup consisted of centrifugation. The attached methodology (Appendix A) contains detailed procedures, which are summarized below.

Assay samples were prepared by spiking known volumes of human plasma with a known amount (constant over all samples in a run) of sisomicin internal standard (IS). Standard curve samples were generated by spiking interference free human plasma samples with known amounts of gentamicin and paromomycin (as free bases) and IS. These standard curve and assay samples were prepared for analysis, then 10 µl aliquots were injected onto the HPLC column for chromatographic separation and subsequent fluorometric detection of drug and IS peaks. The peak height ratios of gentamicin (free base) to IS and paromomycin (free base) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Next, standard curve concentrations and gentamicin (free base) to IS or paromomycin (free base) to IS peak height ratios of the standard curve samples were fit by weighted least squares linear regression to the equation for the best straight line, $y = mx + b$, where y = peak height ratio and x = drug (free base) concentrations. Finally, drug concentrations in assay samples were calculated for each run by this equation from the gentamicin (free base) to IS or paromomycin (free base) to IS peak height ratios obtained by HPLC.

Plasma samples for analysis were thawed and mixed by vortexing (if appropriate), then pipetted (0.2 ml) into glass culture tubes. A constant amount (440 ng sisomicin in 20 µl of mobile phase) of IS and 20 µl of perchloric acid are added. Upon centrifugation, the resulting supernatant was transferred to a WISP vial and injected onto the column.

The assay described in the report dated January, 7, 1997, requires 0.2 ml plasma samples to determine the concentrations of gentamicin and paromomycin. The method involves centrifugation of plasma with injection of supernatant, separation on a C18 column with an aqueous mobile phase in an isocratic elution, a post column reaction and fluorescence detection. The advantages of this method include a clean baseline, a short run time, small sample size and a very simple sample preparation procedure.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a

mechanism also retains considerable amounts of other lipophilic substances, thereby limiting the LLOQ to 100 µg/ml.

By use of the superior capability of fluorescence detection, the concentrations of gentamicin and paromomycin free base can be quantitatively and reliably measured in human and rat plasma samples. The drugs and IS are baseline separated, and no interfering peaks were observed. The assay was demonstrated to be linear within the concentration ranges of the standard curves, 0.100 to 12 µg/ml for gentamicin and paromomycin as free bases. The CV (and corresponding RE) results of human plasma precision validation for gentamicin ranged from 4.34 to 8.42% (-3.20 to 0.800%, n=12) interday and 2.30 to 3.40% (-7.00 to -1.00%, n=6) intraday and for paromomycin ranged from 5.86 to 9.00% (-4.50 to +2.00%, n=12) interday and 3.12 to 3.96% (-7.50 to +1.87%, n=6) intraday. Mean calculated human plasma concentration results of replicate analyses (n=6) of samples spiked at the LLOQ (100 µg/ml for gentamicin and paromomycin) were 0.107 µg/ml (7.88% CV and +7.00% RE) interday and 0.0851 µg/ml (15.7% CV and -14.9% RE) intraday samples for gentamicin and 0.0960 µg/ml (11.3% CV and -4.00% RE) interday and 0.0867 µg/ml (9.92% CV and -13.3% RE) intraday samples for paromomycin. The signal to noise ratio was better than 2 to 1 for these LLOQ samples. The average recoveries from human plasma were 94.0% for gentamicin and 93.8% for paromomycin for the four concentrations within the standard curve quantitation limits. Stability test results indicate gentamicin and paromomycin are sufficiently stable in 1) prepared human plasma samples to withstand room temperature storage for at least 3 days, 2) human plasma at -70°C to permit storage without significant degradation for up to 1 year and at -20°C to permit storage without significant degradation for no longer than 1 month, 3) human plasma to withstand room temperature storage for at least 6 hours without significant degradation, and 4) human plasma to withstand 5 cycles of repeated freezing (to -70°C) and thawing without significant degradation. In the analyses of replicate (n = 5) blind samples the CV (and corresponding RE) of the results for four concentrations within the standard curve quantitation limits for gentamicin ranged 3.35-8.35% (-25.9 to +0.5%) and for paromomycin ranged 2.97-8.16% (-3.27 to +2.50%).

Short Validation Results: Gentamicin free base in rat plasma

Lower Limit of Quantitation:	0.100 µg/ml
Interday Mean, CV and RE:	0.0992 µg/ml, 4.29% and -0.800%
Intraday Mean, CV and RE:	0.0940 µg/ml, 5.42% and -6.00%
Standard curve range:	0.100-12.0 µg/ml
Interday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	3.51 to 8.80%
RE Range:	-5.93 to +4.96%
Intraday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	1.92 to 4.63%
RE Range:	-9.27 to +2.83%
Overall Mean Recovery:	93.4%

Short Validation Results: Paromomycin free base in rat plasma

Lower Limit of Quantitation:	0.100 µg/ml
Interday Mean, CV and RE:	0.0924 µg/ml, 10.8% and -7.57%
Intraday Mean, CV and RE:	0.0864 µg/ml, 8.08% and -13.7%
Standard curve range:	0.100-12.0 µg/ml
Interday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	6.30 to 12.4%
RE Range:	-4.90 to +10.6%
Intraday Precision Concentrations:	0.200, 0.800, 2.50, and 5.00 µg/ml
CV Range:	8.64 to 15.4%
RE Range:	-9.07 to +6.52%
Overall Mean Recovery:	68.2%

Study Description: Gentamicin and Paromomycin in Rat Plasma

The CV (and corresponding RE, for n=6) results of rat plasma precision validation for gentamicin ranged from 3.51 to 8.80% (-5.93 to +4.96%) interday and 1.92 to 4.63% (-9.27 to +2.83%) intraday and for paromomycin ranged from 6.30 to 12.4% (-4.90 to +10.6%) interday and 8.64 to 15.4% (-9.07 to +6.52%) intraday. Mean calculated rat plasma concentration results of replicate analyses of samples spiked at the LLOQ (100 µg/ml for gentamicin and paromomycin) were 0.0992 µg/ml (4.29% CV and -0.800% RE, n=3) interday and 0.0940 µg/ml (5.42% CV and -6.00% RE, n=6) intraday samples for gentamicin and 0.0924 µg/ml (10.8% CV and -7.57% RE, n=3) interday and 0.0864 µg/ml (8.08% CV and -13.7% RE, n=6) intraday samples for paromomycin. The signal to noise ratio was better than 2 to 1 for these LLOQ samples. The average recoveries from rat plasma were 93.4% for gentamicin and 68.2% (which is noticeably lower than in human plasma) for paromomycin for the four concentrations within the standard curve quantitation limits.

Study Report 25: Pyridostigmine in Human Plasma

Study Characteristics: Study Report 25

Test Article:	Pyridostigmine
Test System:	human plasma
Internal Standard:	Neostigmine bromide
Sample Assay Volume:	0.5 ml
Sample Cleanup:	protein precipitation

Analytical System

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5μ particle size
Mobile Phase:	CH_3CN/H_2O (1:1, v/v) with 0.05% TMACl, 5 mM $(NH_4)_2HPO_4$ (final concentrations) apparent pH = 7.2

Validation Results: pyridostigmine cation in human plasma

Lower Limit of Quantitation:	1.53 ng/ml
Interday Mean, CV and RE:	1.43 ng/ml, 12.3% and -6.32%
Intraday Mean, CV and RE:	1.63 ng/ml, 14.6% and 6.72%
Standard curve range:	1.53 to 76.3 ng/ml
Interday Precision Concentrations:	3.05, 9.16, 30.5, and 45.8 ng/ml
CV Range:	3.24 to 17.1%
RE Range:	-0.109 to +3.55%
Intraday Precision Concentrations:	3.05, 9.16, 30.5, and 45.8 ng/ml
CV Range:	30.6 to 9.75%
RE Range:	-6.19 to -1.00%
Blind Sample Assay	
Concentration Range:	2.71 to 24.33 ng/ml
RE Range:	+3.16 to +12.18%
Overall Mean Recovery:	66.8%
Stability	
Plasma Freezer Storage:	-80°C for 135 days -20°C for 6 days
Processed Sample:	Room temp. for 6 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Study Description: Pyridostigmine in Human Plasma

Pyridostigmine bromide is a dimethyl carbamic ester of 3-hydroxy-1-methyl pyridinium bromide (Figure 1). Pyridostigmine is the drug of choice in the treatment of myasthenia gravis[71,72,73,74] and is useful in anesthesiology as an antagonist to reverse non-depolarizing muscle relaxants.[75 and 76] Pyridostigmine pretreatment also appears to be a promising adjunct to routine care in the management of organophosphate poisoning.[77and 78] Because of its greater effectiveness, much longer duration of action and, most importantly, fewer and lower degree of muscarinic side effects, pyridostigmine is preferred over other acetylcholinesterase inhibitors.[79,80,81]

Previous methods developed in the past few years for the determination of pyridostigmine in biological fluids include spectrophotometry, paper radiochromatoelectrophoresis, gas-liquid chromatography and high-performance liquid chromatography (HPLC). The spectrophotometric assay is usually not sensitive enough to detect concentrations of pyridostigmine below 0.5 µg/ml, and other substances in biological fluids often interfere with the assay.[82and 83] Paper radiochromatoelectrophoretic techniques are time-consuming and suffer from a lack of sensitivity, and radioactive compounds are difficult to use in human studies.[84,85,86] Gas-liquid chromatographic methods are unsuitable for routine measurements, because of the great expense and poor reproducibility of current methods.[87,88,89,90,91,92] Other assays[93,94,95,96] include a reverse-phase HPLC procedure for the analysis of pyridostigmine in plasma developed in this laboratory.[97] This report presents additional validation data for a superior method developed in this laboratory that employs an aqueous mobile phase and a silica gel column.[98]

Plasma samples were analyzed for pyridostigmine (cation) with an HPLC procedure that uses a silica gel column, an (acetonitrile/water) aqueous mobile phase, UV absorbance detection, and a 0.5 ml method sample size. Sample cleanup consisted of plasma protein precipitation with acetonitrile and centrifugation and a C8 solid phase extraction followed by elution with an acetonitrile/dibasic ammonium phosphate solution. The attached method SOP (Appendix A) contains detailed procedures and results, which are summarized below.

Assay samples were prepared by spiking known volumes of human plasma with a known amount (constant over all samples in a run) of neostigmine bromide internal standard (IS). Standard curve samples were generated by spiking interference free human plasma samples with known amounts of pyridostigmine (cation) and IS. These standard curve and assay samples were prepared for analysis, then 25 to 50 µl aliquots were injected onto the HPLC column for chromatographic separation and subsequent UV absorbance detection of drug and IS peaks. The peak height ratios of

pyridostigmine (cation) to IS were calculated for each sample from the measured peak heights obtained by HPLC. Next, standard curve concentrations and pyridostigmine (cation) to IS peak height ratios of the standard curve samples were fit by weighted least squares linear regression to the equation for the best straight line, y = mx + b, where y = peak height ratio and x = pyridostigmine (cation) concentrations. Finally, drug concentrations in assay samples were calculated for each run by this equation from the pyridostigmine (cation) to IS peak height ratios obtained by HPLC.

Plasma samples for analysis are thawed and mixed by vortexing (if appropriate), then pipetted (0.5 ml) into glass culture tubes. A constant amount (18.75 µg neostigmine bromide) of the internal standard and 1 ml of CH_3CN to precipitate plasma proteins is added. Upon centrifugation the resulting supernatants were poured onto prewashed C8 Bond Elut cartridges, which were then rinsed with 2 ml water, 4 ml of CH_3CN/H_2O (50:50), 2 ml of 100% CH_3CN, and 0.5 ml of CH_3CN/H_2O (85:15) containing 1 mM $(NH_4)_2 HPO_4$ at pH 3.6. The fraction containing pyridostigmine was eluted by adding 2 ml of CH_3CN/H_2O (85:15) containing 1 mM $(NH_4)_2HPO_4$ at pH 3.6 to the C8 cartridges. The eluates were concentrated by evaporation to approximately 200 µl under nitrogen at room temperature (if samples are evaporated too much or to dryness, eluting solution is added back), transferred to WISP vials, and injected onto the column.

The assay described in the report dated November, 22, 1995, as amended March, 29, 1996, requires 0.5 ml plasma samples to determine the cation concentrations of pyridostigmine. The method involves precipitation of plasma proteins, a C8 solid phase extraction step with acidified 85% acetonitrile, separation on a silica gel column with an aqueous mobile phase in an isocratic elution, and ultraviolet absorbance detection. HPLC assays for basic amine drugs in biological samples that make use of a silica gel column and an aqueous mobile phase have been operated in this laboratory for 10 years [29 - 31].[99,100,101] The advantages of this method over the method previously developed (a reverse phase method) include a cleaner baseline and a shorter run time.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange[102] and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The

interfering substances in biological fluids elute at the solvent front, leaving a very clean baseline around the retention time of the drug. In this method, the mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica. Overnight saturation of mobile phase prior to use is beneficial to the whole system, since silica gel slowly dissolves in neutral aqueous solution and the water flowing through the silica gel approaches the equilibrium concentration of silica. The saturated mobile phase does not dissolve silica from the analytical columns and degradation of the analytical column is decreased relative to the single pass system. During the analysis, the pH of mobile phase is maintained at 7.2. A higher pH shortens the drug retention time and distorts the separation.

By use of protein precipitation and a solid phase extraction step for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of ultraviolet detection, the cation concentration of pyridostigmine can be quantitatively and reliably measured in human plasma samples. The drug and IS are baseline separated, and no interfering peaks were observed. The assay was demonstrated to be linear within the range of the standard curve, 1.53 to 76.3 ng/ml pyridostigmine cation. The coefficients of variation (CV's) of results for plasma precision validation ranged from 3.24 to 17.1% interday and 3.06 to 9.75% intraday, while percent error of measured results compared to spiked values ranged -0.109 to 3.55% interday and -6.19 to -1.00% intraday for pyridostigmine (cation). The LLOQ of the assay is 1.53 ng/ml for the cation concentrations of the drug in plasma where the signal to noise ratio is better than 3 to 1, the interday CV (n = 6) is 12.0% and error is -6.32% and the intraday CV (n = 6) is 14.6% and error is +6.72%. From plasma, the average (four concentrations) mean (n = 3) recovery for pyridostigmine (cation) was 66.8%. Stability test results indicate pyridostigmine (cation) is sufficiently stable in 1) prepared plasma samples to withstand room temperature storage for at least 6 days, 2) plasma at -80°C to permit storage without significant degradation for up to 135 days and sufficiently stable in plasma at -20°C to permit storage without significant degradation for up to 6 days, 3) plasma to withstand room temperature storage for at least 6 hours without significant degradation, and 4) plasma to withstand 5 cycles of repeated freezing and thawing without significant degradation. The CVs of the results for the analyses of blind pyridostigmine (cation) samples (n = 7) at four concentrations within the standard curve limits ranged 6.06-18.11% while error ranged 3.16 to 12.18%.

Study Report 26: WR 242511 in Human and Dog Plasma

Study Characteristics: Study Report 26

Test Article:	WR 242511
Test System:	human plasma dog plasma
Internal Standard:	Chlorpheniramine maleate
Sample Assay Volume:	0.5 ml
Sample Cleanup:	methyl *t*-butyl ether extraction

Analytical System

Detector:	UV at 350 nm
Column Type:	silica
Column Size:	4.6x250 mm, 5µ particle size
Mobile Phase:	CH_3CN/H_2O (7:3, v/v) with 0.008% TEA and 0.005% H_3PO_4 (final concentrations)

Validation Results: WR 242511 free base in human plasma

Lower Limit of Quantitation:	4.00 ng/ml
Interday Mean, CV and RE:	4.57 ng/ml, 5.84% and 14.2%
Intraday Mean, CV and RE:	3.69 ng/ml, 7.87% and -15.6%
Standard curve range:	4.00 to 1024 ng/ml
Interday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	8.74 to 11.9%
RE Range:	-3.32 to +5.40%
Intraday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	2.99 to 5.90%
RE Range:	+5.21 to +12.3%
Blind Sample Assay	
Concentration Range:	4.70 to 822 ng/ml
RE Range:	-6.27 to +21.9%
Overall Mean Recovery:	77.1%
Stability	
Plasma Freezer Storage:	-70°C for 6 months -20°C for 6 months
Processed Sample:	Room temp. for 4 days
Plasma Storage:	Room temp. for 6 hours
5 Cycle Freeze/Thaw:	5 cycles to -70°C
Standard Solution:	6 months

Short Validation Results: WR 242511 free base in dog plasma

Lower Limit of Quantitation:	4.00 ng/ml
Precision Mean, CV and RE:	4.42 ng/ml, 6.52% and +10.4%
Standard curve range:	4.00 to 1024 ng/ml
Interday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	4.14 to 13.3%
RE Range:	-5.78 to -0.104%
Intraday Precision Concentrations:	8.00, 32.0, 128, and 256 ng/ml
CV Range:	0.764 to 5.12%
RE Range:	-10.8 to -3.35%
Overall Mean Recovery:	79.3%

Study Description: WR 242511 in Human and Dog Plasma

This report describes a high performance liquid chromatographic (HPLC) assay and provides data validating the assay for a compound of the 8-aminoquinoline class. The compound, 8-[(4-amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline (DL) tartrate (WR 242511, Figure 1), holds promise[103] in an effort to replace primaquine, the radical cure and prophylaxis for vivax and ovale malaria and is being developed by WRAIR as an anti-cyanide drug.

Assays for other 8-aminoquinolines include high performance liquid chromatography (HPLC) methods with electrochemical,[104] ultraviolet,[105] and fluorescence[106] detection. An HPLC method with oxidative electrochemical detection has been described[107] for WR 242511 in 0.25 ml plasma samples with a detection limit of 10 ng/ml. This report presents validation data for a superior method that employs an aqueous mobile phase, an unbonded silica gel column[108] and ultraviolet detection for WR 242511 free base concentration determinations in 0.5 ml human and dog plasma samples with a lower limit of quantitation of 4 ng/ml.

Plasma samples were analyzed for WR 242511 free base with an HPLC procedure that uses a silica gel column, an (acetonitrile/water) aqueous mobile phase, UV absorbance detection, and a 0.5 ml method sample size. Sample cleanup consisted of extraction into methyl *t*-butyl ether. The attached methodology (Appendix A) contains detailed procedures, which are summarized below.

Assay samples were prepared by spiking known volumes of human plasma with a known amount (constant over all samples in a run) of CPA internal standard (IS). Standard curve samples were generated by spiking a known amount of WR 242511 tartrate into interference free human plasma which is then brought to a known volume, divided by serial dilution and

spiked with a known amount of IS. These standard curve and assay samples were prepared for analysis, then 40 µl aliquots were injected onto the HPLC column for chromatographic separation and subsequent UV absorbance detection of drug and IS peaks. The peak height ratios of WR 242511 to IS were calculated for each sample from the measured peak heights obtained by HPLC. Next, standard curve concentrations and WR 242511 to IS peak height ratios of the standard curve samples were fit by 1/y weighted least squares linear regression to the equation for the best straight line, $y = mx + b$, where y = peak height ratio and x = WR 242511 free base concentrations. Finally, drug concentrations in assay samples were calculated for each run by this equation from the WR 242511 to IS peak height ratios obtained by HPLC.

Stock solutions of WR 242511 tartrate and chlorpheniramine maleate internal standard (IS) were stored in a 4°C refrigerator and protected against exposure to light as necessary, and checked for deterioration by following the ratio of drug to internal standard peak heights in a diluted solution (solutions are discarded when a more than 10% change in the ratio is observed or by 2 months after the preparation date).

Plasma samples for analysis were thawed and mixed by vortexing (if appropriate), then pipetted (0.5 ml) into glass culture tubes. A constant amount (1.0 µg chlorpheniramine maleate) of IS, 100 µl of 0.1N NaOH, and 3 ml of methyl *t*-butyl ether are added. Upon centrifugation and freezing of the aqueous layer, the resulting supernatant was transferred to a clean tube, evaporated to dryness, reconstituted in 70% acetonitrile, transferred to WISP vials, and injected onto the column.

The assay described in the report dated December, 12, 1996, requires 0.5 ml plasma samples to determine the concentrations of WR 242511 free base. The method involves extraction from plasma with methyl *t*-butyl ether, separation on a silica gel column with an aqueous mobile phase in an isocratic elution, and ultraviolet absorbance detection. The advantages of this method include a clean baseline and a short run time.

The reversed-phase system (alkyl bonded silica gel with an aqueous mobile phase) is the most widely used HPLC technique in assays for drugs in biological fluids. In this kind of a system, the retention mechanism depends mainly on the lipophilic character of substances to be analyzed. Such a mechanism also retains considerable amounts of other lipophilic substances, thereby interfering with the drug peak. On the other hand, in a system consisting of a bare silica gel and an aqueous mobile phase, the retention mechanism results mainly from ion exchange[6] and only partially from lipophilic interactions. Thus, endogenous non-ionic neutral lipid compounds and anionic compounds will not be retained on the silica gel column; only the cationic (e.g. ammonium) ions will be retained. The interfering substances in biological fluids elute at the solvent front, leaving a

very clean baseline around the retention time of the drug. In this method, the mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica. Overnight saturation of mobile phase prior to use is beneficial to the whole system, since silica gel slowly dissolves in neutral aqueous solution and the water flowing through the silica gel approaches the equilibrium concentration of silica. The saturated mobile phase does not dissolve silica from the analytical columns and degradation of the analytical column is decreased relative to the single pass system.

By use of a solvent extraction step for sample clean-up, an unbonded silica gel column combined with an aqueous mobile phase for separation, and the superior capability of ultraviolet detection, the concentration of WR 242511 free base can be quantitatively and reliably measured in human and dog plasma samples. The drug and IS are baseline separated, and no interfering peaks were observed. The assay was demonstrated to be linear within the range of the standard curve, 4.00 to 1024 ng/ml WR 242511 free base. The CVs of results for human plasma precision validation ranged from 8.74 to 11.9% interday and 2.99 to 5.90% intraday, while percent RE of measured results compared to serially diluted concentrations ranged -3.32 to +5.40% interday and +5.21 to +12.3% intraday for WR 242511 free base. The mean concentrations (n = 6) obtained for human plasma samples serially diluted to the LLOQ (4.00 ng/ml) were 4.57 ng/ml interday (5.84% CV and +14.2% RE) and 3.38 ng/ml intraday (7.87% CV and -15.6% RE) where the signal to noise ratio was better than 3 to 1. WR 242511 average recovery from human plasma extraction for the four concentrations within the standard curve quantitation limits was 77.1%. Stability test results indicate WR 242511 is sufficiently stable in 1) human plasma samples prepared for assay (includes extraction, evaporation, and reconstitution in 70% acetonitrile) to withstand room temperature (RT) storage for at least 4 days, 2) human plasma at -70°C and at -20°C to permit storage without significant degradation for up to 6 months, 3) human plasma to withstand RT storage for at least 6 hours without significant degradation, and 4) human plasma to withstand 5 cycles of repeated freezing in a -70°C freezer and thawing at room temperature without significant degradation. The CVs of the results for the analyses of blind WR 242511 human plasma samples (n = 5) at five concentrations within the standard curve limits ranged 0.958-9.61% while REs ranged -8.39 to +21.9%.

The CV (and corresponding RE, for n = 6) results of dog plasma precision validation for WR 242511 ranged from 4.14 to 13.3% (-5.78 to -0.104%) interday and 0.764 to 5.12% (-10.8 to -3.35%) intraday. Mean back calculated dog plasma concentration results of replicate analyses of precision standard curve samples serially diluted to the LLOQ (4.00 ng/ml for WR 242511 free base concentration) was 4.42 ng/ml (6.52% CV and +10.4% RE, n = 4). The signal to noise ratio was better than 3 to 1 for these LLOQ samples. WR 242511 average recovery from dog plasma extraction for the four concentrations within the standard curve quantitation limits was 79.3%.

Study Report No. 27: WR 238,608 R and S Isomers in Human Plasma

Study Characteristics: Study Report 27

Test Article:	WR 238,608 (R isomer) WR 238,608 (S isomer)
Test System:	human plasma
Internal Standard:	WR 211,789
Sample Assay Volume:	1.00 ml
Sample Cleanup:	methyl *t*-butyl ether double extraction

Analytical System

Detector:	fluorescence-Ex: 375 nm; Em: 480 nm
Column Type:	Chiralcel OD-R, cellulose tris
Column Size:	4.6x250 mm, 10µ particle size
Mobile Phase:	acetonitrile/$NaClO_4$ (45:55, v/v) at pH 5.
Standard curve range:	5.00 to 1000 ng/ml

This project was requested as described in a COR letter dated Oct. 20, 1995. This study will describe development and validation of an assay for WR 238,608 enantiomers in human plasma. Validation of the assay is underway with LLOQ at 5 ng/ml. Standards of the enantiomers requested Oct. 4, 1996 were received on October 24, 1996.

Study Report No. 28: Halofantrine and WR 178460 R&S Isomers in Human Plasma

Study Characteristics: Study Report 28

Test Article:	Halofantrine (R isomer) Halofantrine (S isomer) WR 178460 (R isomer) WR 178460(S isomer)
Test System:	human plasma

This project was requested as described in a COR letter dated Oct. 20, 1995. WR 216062 and WR 216063 standard samples were received October 20, 1995 for use in development and validation of an assay for halofantrine and desbutylhalofantrine enantiomers in human plasma.

Study Report No. 29: Chloroquine and Metabolites in Human Plasma

Study Characteristics: Study Report 29

Test Article:	Chloroquine Monodesethyl Chloroquine Didesethyl Chloroquine
Test System:	human plasma
Analytical System	
Detector:	MS/MS

This highest priority project was requested in a COR letter dated December 6, 1995. Additional standard compounds were received for chloroquine diphosphate (WR 1544), the didesethyl chloroquine metabolite (WR 112472) and the monodesethyl chloroquine metabolite (WR 29623) on October 24, 1996. Method validation is in progress.

Study Report No. 30: WR 243251 in Human Plasma

Study Characteristics: Study Report 30	
Test Article:	WR 243251
Test System:	human plasma
Analytical System	
Detector:	MS/MS

This project was requested in a COR letter dated May 6, 1996. WR 243,251 standard compound was received May 10, 1996. Method development is in progress.

Study Report No. 31: WR 238,608, Mefloquine, Chloroquine, Quinine, and Doxycycline in Dog Plasma

Study Characteristics: Study Report 31

Test Article:	WR 238,608 Mefloquine Chloroquine Quinine Doxycycline
Test System:	dog plasma
Analytical System	
Detector:	MS/MS

A study on WR 238,605 used in combination with mefloquine, chloroquine, quinine and doxycycline in dog plasma as described in a COR letter dated Oct. 20, 1995 (see also WR5/P 95-3) continued.

Study Report No. 32: WR 238,608 in Human Plasma

Study Characteristics: Study Report 32

Test Article:	WR 238,608
Test System:	human plasma
Internal Standard:	Verapamil
Sample Assay Volume:	100 µl
Sample Cleanup:	acetonitrile precipitation

Analytical System

Detector:	MS/MS
Column Type:	hypersil silica
Column Size:	4.6x50 mm, 5µ particle size
Mobile Phase:	acetonitrile/water/TFA (90:10:0.06, v/v/v).

A long term stability study for WR 238,605 as racemate in human blood and plasma (plasma data to 2 months faxed to COR on Oct. 4, 1996) for up to two years requested in a COR letter dated Feb. 15, 1996 continued.

Study Report No. 33: Halofantrine and WR 178460 in Human Plasma and Blood

Study Characteristics: Study Report 33

Test Article:	Halofantrine WR 178460
Test System:	human plasma and blood
Analytical System	
Detector:	MS/MS

Long term (2 year) freezer stability study and LC/MS/MS method development is in progress for halofantrine and desbutylhalofantrine in human plasma and blood.

ROUTINE ASSAY RESULTS

The following section presents short descriptions of specific routine sample assays completed or currently in progress during the contract. Complete data findings are presented in Appendix B.

TABLE 5: ROUTINE ANALYSES PERFORMED

Report Title	Report Status	Test Article	Test System	No. of Results	Report No.	
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Plasma Samples Obtained for the Initial Year of the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 HCl (Halofantrine Hydrochloride) in Rats"	3/31/93 final report	halofantrine WR 178,460	rat plasma	118 118	Hal/P	91-4
Routine Analysis for Halofantrine and WR 178,460 (free bases) in Blood Samples Obtained for the Protocol Titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria"	1/21/92 final report	Halofantrine WR 178,460	human blood	107 107	Hal/B	91-5
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Blood Samples Obtained under the Protocol Titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria"	6/23/92 final report	mefloquine	human blood	107	Mef/B	91-5
Results assoc. with Hal/P 91-1	4/28/92 final report	halofantrine WR 178,460	dog plasma	29 29	Hal/P	91-6
Routine Analysis for Mefloquine (as Free Base) in Plasma Samples Obtained under the Protocol Titled "Evaluation of the Tolerance of Prophylactic Mefloquine Regimens"	3/1/93 final report	mefloquine	human plasma	660	Mef/P	91-7
Study continued as WR6/PU 93-1	8/3/92 data	WR 6026 WR 211,789	plasma	194 194	WR6/P	92-1

TABLE 5: ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Status	Test Article	Test System	No. of Results	Report No.	
Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Plasma Samples Obtained for the Second Year of the Protocol Titled "Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 HCl (Halofantrine Hydrochloride) in Rats, HWA Study No. 193-558"	3/31/93 final report	halofantrine WR 178,460	rat plasma	154 154	Hal/P	92-2
Routine Analysis for WR 238,605 (as free base) of Blood and Plasma Samples Obtained for the Protocol Titled "Rising Single Oral Dose Safety and Tolerance Study of WR 238,605 Succinate"	2/6/95 final report	WR 238,605	human plasma, blood	893 74	WR5/PB	92-3
Routine Analysis for WR 6026, WR 211,789 and WR 254,421 (as free bases) in Plasma and Urine Samples Obtained under the Protocol Titled "Phase II Clinical Trial of Oral WR 6026 2HCl in Patients with Visceral Leishmaniasis - Initial Dose Ranging for Efficacy, Safety and Tolerance"	3/12/93 data	WR 6026 WR 6026 WR 211,789 WR 254,421	human plasma, urine	117 68 68 68	WR6/PU	93-1
Routine analysis for Halofantrine and WR 178,460 (as free bases) of Plasma Samples Obtained for the Protocol Titled "Pharmacokinetics of a New Multiple Dose Halofantrine Regimen"	12/10/96 in review	halofantrine WR 178,460		642 642	Hal/P	93-2
No protocol	2/25/94 data	*p*-aminoheptanophenone	dog plasma	876	Pah/P	93-3

TABLE 5: ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Status	Test Article	Test System	No. of Results	Report No.	
Routine Analysis for WR 238,605 (as free base) of Plasma Samples Obtained for the Protocol Titled "Thirteen Week Oral Toxicity Study of WR 238,605 with a Thirteen Week Recovery Period in Dogs"	4/25/94 in review	WR 238,605	dog plasma	330	WR5/P	93-4
Routine Analysis for WR 238,605 (as free base) of Plasma Samples Obtained for the Protocol Titled "Thirteen Week Oral Toxicity Study of WR 238,605 with a Thirteen Week Recovery Period in Rats"	1/20/94 final report as amended 11/4/96	WR 238,605	rat plasma	154	WR5/P	93-5
Routine Analysis for Primaquine and Carboxyprimaquine of Serum Samples Obtained for the Protocol Titled "Primaquine and Several Recommended Prophylactic Drugs against Falciparum Malaria: Field Trial II"	5/3/96 final report	primaquine carboxy metab	human serum	60	Pri/P	93-6
Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Rat Liver, Bile and Perfusate Samples	10/28/94 final data	halofantrine	rat liver perfsate bile		Hal/lpb	93-7
Routine Analysis for WR 238,605 (as free base) Human Plasma and Blood Samples Obtained for the Protocol Titled "Pharmacokinetics, Pharmacodynamics, Safety and Tolerance of a Single Oral Dose of WR 238605 Succinate"	9/16/94 final data	WR 238,605	human plasma blood	120 120	WR5/PB	93-8

TABLE 5: ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Status	Test Article	Test System	No. of Results	Report No.	
Routine Analysis for *p*-Aminoheptanophenone of Dog Plasma Samples Obtained for the Protocol Titled "*p*-Amino-heptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Dogs"	2/7/95 final data	*p*-aminohep-tanophenone	dog plasma	189	Pah/P	93-9
Routine Analysis for WR 238,605 (as free base)Monkey Plasma Samples	11/22/94 final data	WR 238,605	monkey plasma	12	WR5/P	94-1
Routine Analysis for *p*-Amino-heptanophenone Rat Plasma Samples Obtained for the Protocol Titled "p-Aminohept-anophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Rats"	2/7/95 final data	*p*-aminohept-anophenone	rat plasma	152	Pah/P	94-2
Tentative Title: Routine Analysis for WR 6026 and Metabolites in Plasma and Urine Samples Obtained for the Protocol Titled "Clinical Trial of Oral WR6026•2HCl in Patients with Brazilian Visceral Leishmaniasis due to L. chagasi: Initial Dose Range Determine	1/27/97 final data more samples expected	WR 6026 WR 211789 WR 254421	human plasma urine plasma urine plasma urine plasma	38 37 36 17 24 36 12	WR6/PU	94-3
Tentative Title: Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced P. falciparum Malaria Infection in Healthy Non-immune Subjects: A Dose Ranging Study"	11/21/94 final data	WR 238,605	human plasma blood	28 28	WR5/PB	94-4

TABLE 5: ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Status	Test Article	Test System	No. of Results	Report No.	
Blind sample results to be added to SR 13B, Supplement II	10/12/94 final data	WR 238,605	dog plasma	30	WR5/P	94-5
Routine Analysis for Pyridostigmine (Cation) in Plasma Samples for the Protocol Titled "A Study to Evaluate the Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Pyridostigmine when given in Single and Multiple Doses to Males and Females in Diff	4/3/96 final report	Pyridostigmine	human plasma	2639	Pyr/P	94-6
Tentative Title: Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "A Multiple Dose Safety, Tolerance and Pharmacokinetic Study of WR 238605 when Given to Healthy Male and Female Subjects"	8/29/96 in review	WR 238605	human plasma	709	WR5/P	94-7
Tentative Title: Routine Analysis for WR 238605 in Rat Plasma Samples Obtained for the Protocol Titled "Six Month Oral Toxicity Study of WR 238605 Succinate in Rats	9/17/96 in review	WR 238605	rat plasma	405	WR5/P	95-1
Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "Evaluation of WR 238605 as a Prophylactic Agent Against Induced P. Falciparum Malaria Infection in Healthy Non-Immune Subjects II: A Multiple Dose Causal versus Suppressive	4/24/96 final data in prgrss in prgrss	WR 238605 chloroquine chiral	human plasma blood blood plasma blood	226 226 67 226 226	WR5/P	95-2

TABLE 5: ROUTINE ANALYSES PERFORMED (Continued)

Report Title	Report Status	Test Article	Test System	No. of Results	Report No.	
Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "WR 238605 Multiple Drug Interaction Study in Beagle Dogs"	4/26/96 samples received assay in progress	WR 238605 Mefloquine Chloroquine Quinine Doxycycline Halofantrine	human plasma	111	WR5/P	95-3
Routine Analysis for Halofantrine and WR 178460 in Plasma Samples Obtained for the Protocol Titled "Halofantrine as Prophylaxis against Malaria: Multiple-Dose Safety, Tolerance and Pharmacokinetics Study"	1/3/97 final data chiral assay in progress	Halofantrine WR 178,460 Halofantrine WR 178,460	human plasma	1060 1060 1060 1060	Hal/P	95-4
Routine Analysis for Halofantrine and WR 178460 in Aotus Monkey Blood Samples	6/4/96 samples received assay in progress	Halofantrine	monkey blood	165	Hal/B	96-1
Tentative Title: Routine Analysis for WR 238605 in Plasma Samples Obtained for the Protocol Titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of Plasmodium vivax Infection in Thailand"	9/25/96 samples received assay in progress	WR238605	human plasma blood	266 266	WR5/BP	96-2
Tentative Title: Routine Analysis for Gentamicin and Paromomycin in Human Plasma Samples	12/18/96 final data more samples expected	Gentamicin Paromomycin	human plasma	36 47	Gnt/P	96-3

p-Aminoheptanophenone (PAHP, WR 269,410) *p*-Aminooctanophenone (PAOP, WR 258,948) *p*-Aminopropiophenone (PAPP, WR 302)

Pah/P 93-3 Data was presented in the Midterm Report dated August 14, 1994.

Results will be reported in Analysis Report No. 93-3. Status of samples received is described in the table below. Report completion requires completion of method validation.

No. of Samples	Description	Date Received	Status
106	dog plasma	3/3/93	Results Faxed to COR 9/23/93
52	dog blood	3/3/93	Not to be assayed
645	dog plasma	9/21/93	Results Faxed to COR 2/25/94
36	blind spiked dog plasma	9/30/93 11/2/93	Results Faxed to COR 1/25/94
125	dog plasma	10/21/93	Results Faxed to COR 2/25/94

Pah/P 93-9

Samples (189 dog plasma) were received July 12, 1994 to be analyzed in accordance with the protocol titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Dogs." Analysis is complete and final results were Faxed to the COR on February 7, 1995. Report completion requires completion of the method validation report.

Pah/P 94-2

Samples (152 rat plasma) were received July 12, 1994 to be analyzed in accordance with the protocol titled "*p*-Aminoheptanophenone (PAHP) (WR269410) Single Dose IV and Oral Pharmacokinetic, Pharmacodynamic, Bioavailability and Metabolism Study in Rats." Analysis is complete and final results were Faxed to the COR on February 7, 1995. Report completion requires completion of the method validation report.

HALOFANTRINE

Hal/P 91-4: Data was presented in the Midterm Report dated August 14, 1994.

Analysis Report Nos. Hal/P 92-2 initially titled "Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Plasma Samples Obtained under the Protocol Titled 'Combined Chronic Toxicity and Oncogenicity

Study of WR-171,669 HCl (Halofantrine Hydrochloride) in Rats, HWA Study No. 193-558'" and Hal/P 91-4 from the same protocol were completed and revised Analysis Report Nos. Hal/P 92-2 and Hal/P 91-4 were approved by the COR as final on June 30, 1993.

Analysis of 118 rat plasma samples for determination of the free base concentrations of halofantrine (WR 171,669) and of its metabolite (WR 178,460) was accomplished by use of an HPLC method described in Study Report No. 17, dated April 25, 1990 and developed under contract DAMD17-86-C-6150. The samples were obtained from Hazleton Laboratories America, Inc., in accordance with the protocol titled "Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 HCl (Halofantrine Hydrochloride) in Rats." Analytical results are presented in the report dated March 31, 1993, for plasma samples from the initial year of a 2 year study of rats given daily oral gavage doses of halofantrine hydrochloride and were obtained from analyses performed from May 29 through June 20, 1991.

Hal/P 91-5: Data was presented in the Midterm Report dated Aug. 14, 1994.

Analysis Report Hal/91-5, titled "Routine Analysis for Halofantrine and WR 178,460 (as Free Bases) of Blood Samples Obtained under the Protocol Titled 'Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria,'" described the routine analysis of 107 human blood samples for the determination of the halofantrine free base concentrations, and was submitted to the COR on January 21, 1992 for review and approved by the COR as final on July 31, 1993.

Hal/P 91-6: Data was presented in the Midterm Report dated Aug. 14, 1994.

Results associated with Hal/P 91-1. No further action is planned for this routine assay.

Hal/P 92-2: Data was presented in the Midterm Report dated Aug, 14, 1994.

Analysis Report Nos. Hal/P 92-2 initially titled "Routine Analysis for Halofantrine and WR 178,460 (as free bases) of Plasma Samples Obtained under the Protocol Titled 'Combined Chronic Toxicity and Oncogenicity Study of WR-171,669 HCl (Halofantrine Hydrochloride) in Rats, HWA Study No. 193-558'" and Hal/P 91-4 from the same protocol were completed and revised Analysis Report Nos. Hal/P 92-2 and Hal/P 91-4 were approved by the COR as final on June 30, 1993.

Hal/P 93-2

Final results of the routine analysis of 339 human plasma samples received March 3, 1993 and 303 human plasma samples received April 14, 1993 for halofantrine and WR 178,460 (free base) concentrations for samples

obtained under the protocol "Pharmacokinetics of a New Multiple Dose Halofantrine Regimen" faxed to the COR July 7, 1993 and enclosed in Quarterly Report No. 6.

Analysis of 642 human plasma samples for determination of the free base concentrations of halofantrine (WR 171,669) and of its metabolite (WR 178,460) was accomplished by use of an HPLC method described in Study Report No. 17, developed under contract DAMD17-86-C-6150. The samples were obtained from the South Florida Drug Research Corporation, Inc., in accordance with the protocol titled "Pharmacokinetics of a New Multiple Dose Halofantrine Regimen." Analytical results are presented in the report dated December 10, 1996, for plasma samples from human male subjects from analyses performed from April 30 through June 8, 1993.

Hal/lpb 93-7

Final bile, liver and perfusate results were attached to Quarterly Report 11 and appear in Appendix B. Additional data, showing just perfusate extraction results, were faxed to the COR December 28, 1994. Analysis Report Hal/Lprb 93-7 and supplements to the human validation report are now in preparation. Remaining samples were returned to WRAIR on July 25, 1995. Report completion requires completion of method validation reports.

Hal/P 95-4

Samples are to be analyzed in accordance to the protocol titled "Halofantrine as Prophylaxis against Malaria: Multiple-Dose Safety, Tolerance and Pharmacokinetics Study." Final data on 1060 samples for halofantrine and WR 178,460 (free base) concentrations were faxed to the COR on January 3, 1997. A chiral assay of the same samples is currently in progress.

Hal/B 96-1

Monkey blood samples (165) to be analyzed for halofantrine were received on June 4, 1996. No protocol is available for this study.

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

Mef/91-5: Data was presented in the Midterm Report dated August 14, 1994.

Analysis of 107 human blood samples for determination of the free base concentration of mefloquine (WR 171,669) was accomplished by use of an HPLC method described in Study Report No. 19, dated January 14, 1992 and developed under contract DAMD17-86-C-6150. The samples were obtained from the Armed Forces Research Institute of Medical Sciences and the Royal

Thai Army, in accordance with the protocol titled "Efficacy of Halofantrine and Mefloquine in the Treatment of Falciparum Malaria." Analytical results are presented in the report dated June 23, 1992, for blood samples from humans given halofantrine (1500 mg) and/or mefloquine (750 mg) and were obtained from analyses performed April 17-21, 1992.

Mef/91-7: Data was presented in the Midterm Report dated Aug. 14, 1994.

Analysis of 660 human plasma samples for determination of the free base concentration of mefloquine hydrochloride (WR 142,490), was accomplished by use of the HPLC method described in Study Report No. 14B dated August 29, 1989 under contract DAMD17-86-C-6150. The samples were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, in accordance with the protocol titled "Evaluation of the Tolerance of Prophylactic Mefloquine Regimens." Analytical results are presented in the report dated March 1, 1993, for plasma samples from humans given mefloquine hydrochloride and were obtained from analyses performed January 27-April 13, 1992.

Primaquine and its carboxy metabolite

Pri/P 93-6

Samples (60 human serum) were received August 18, 1993 for analysis under the protocol (received July 29, 1993) titled "Primaquine and Several Recommended Prophylactic Drugs against Falciparum Malaria: Field Trial II." Preliminary results were faxed to the COR on June 6, 1994.

Analysis of 60 human serum samples for determination of the concentrations of primaquine (WR 002,975) free base and its carboxylated metabolite (WR 249,725) was accomplished by use of an HPLC method described in Study Report No. 23, dated April 29, 1996 and developed under contract DAMD17-92-C-2028. The samples were obtained from the Walter Reed Army Institute of Research in accordance with the protocol titled "Primaquine and Several Recommended Prophylactic Drugs Against Falciparum Malaria: Field Trial II." Analytical results are presented in the report for serum samples from male and female children from analyses performed from April 4-15, 1994.

WR 238,605

WR5/P 92-3: Data was presented in the Midterm Report dated Aug. 14, 1994.

Analysis Report WR5/BP 92-3 was submitted for review on July 7, 1994 and a revised report dated February 6, 1995 was accepted as final with changes suggested in a COR letter dated December 16, 1994.

Analysis of 893 human plasma and 74 human blood samples for determination of the WR 238,605 (free base) (the salt is 8-[(4-amino-1-methylbutyl)amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy)quinoline succinate) concentration was accomplished by use of an HPLC method described in Study Report No. 13, dated March 29, 1989 and developed under contract DAMD17-86-C-6150. The samples were obtained from the South Florida Drug Research Corporation according to the protocol titled "Rising, Single Oral Dose Safety and Tolerance Study of WR 238605 Succinate." The analytical results for plasma and blood samples from human subjects given single oral doses of WR 238,605 (succinate) or placebo were obtained from analyses performed between 12/12/92 and 4/28/94 and are presented in the report dated February 6, 1995.

WR5/P 93-4: Data was presented in the Midterm Report dated August 14, 1994.

Results of the analysis of 330 dog plasma samples received on 7/14/93 were reported in Routine Analysis Report WR5/P 93-4, which was submitted for review on April 25, 1994. The protocol for this study is titled "Thirteen week oral toxicity study of WR 238605 with a thirteen week recovery period in dogs." Study Report 13B, Supplement II for dog plasma validation was submitted for review on April 11, 1994 and is being modified as requested in a COR letter dated January 31, 1995.

Analysis of 320 dog plasma samples for determination of the free base concentration of WR 238,605 was accomplished by use of an HPLC method described in Study Report No. 13 Dog Plasma Assay Supplement, dated April 11, 1994 and developed under contract DAMD17-92-C-2028. The samples were obtained from the University of Illinois at Chicago, in accordance with the protocol titled "Thirteen Week Oral Toxicity Study of WR 238605 with a Thirteen Week Recovery Period in Dogs." Analytical results are presented in the report dated April 25, 1994, for plasma samples from male and female dogs from analyses performed from September 30, 1993 through January 11, 1994.

WR5/P 93-5

Analysis of 154 rat plasma samples for determination of the free base concentration of WR 238,605 was accomplished by use of an HPLC method described in Study Report No. 13 Rat Plasma Assay Supplement, dated January 20, 1994 and developed under contract DAMD17-92-C-2028. The samples were obtained from the University of Illinois at Chicago, in accordance with the protocol titled "Thirteen Week Oral Toxicity Study of WR 238605 with a Thirteen Week Recovery Period in Rats." Analytical results are presented in the report dated January 20, 1994, as amended November 4, 1996, for plasma samples from male and female rats from analyses performed from October 12 through 21, 1993.

WR5/P 93-8

Routine analysis of 359 human plasma and 359 human blood samples was completed for Analysis Report WR5/BP 93-8 for samples received in accordance with the protocol titled "Pharmacokinetics Pharmacodynamics, Safety and Tolerance of a Single Oral Dose of WR 238605 Succinate." Final data was attached to Quarterly Report 11. Repeat analysis of selected samples, as requested in a FAX from the COR dated December 9, 1994, was completed and results were faxed to the COR February 7, 1995. A report is in preparation.

WR5/P 94-1

Monkey blood (12) samples were received September 15, 1994. Final analytical results were faxed to the COR on 11/22/94. A brief letter reporting results and referring to the human validation report as suggested by the COR at the April 3, 1995 site visit is in preparation. The analysis was set to proceed with use of blank human plasma for standard curve and control samples and blank monkey plasma as duplicate controls.

WR5/P 94-4

Human plasma (28) and human blood (28) samples were received October 26, 1994 and assayed in accordance with the protocol titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-immune Subjects: A Dose Ranging Study." Final analytical results were faxed to the COR on 11/21/94 and the report is in preparation.

WR5/P 94-5

Results for study WR5/P 94-5 on the analysis of 30 blind dog plasma samples received September 15, 1994 were submitted October 12, 1994 and a statistical analysis was attached to Quarterly Report 11. Data is to be added to Study Report 13B, Supplement II.

WR5/P 94-7

Final results for 709 human plasma samples assayed in accordance with the protocol titled "A Multiple Dose Safety, Tolerance and Pharmacokinetic Study of WR 238605 when given to Healthy Male and Female Subjects" were Faxed to the COR on 7/22/96. Draft Analysis Report WR5/P 94-7 was submitted August 29, 1996 to the COR for review.

WR5/P 95-1

Samples were analyzed in accordance with the protocol titled "Six Month Oral Toxicity Study of WR238605 Succinate in Rats." Rat plasma samples

(325) were received September 21, 1995 and (80) January 30, 1996. Final results were faxed to the COR on September 12, 1996 (enclosed). Draft Analysis Report WR5/P 95-1 was submitted September 17, 1996 to the COR for review.

WR5/P 95-2

Final results were faxed to the COR on April 24 and 30, 1996 for samples analyzed in accordance to the protocol titled "Evaluation of WR 238605 as a Prophylactic Agent against Induced *P. falciparum* Malaria Infection in Healthy Non-Immune Subjects II: A Multiple-Dose Causal versus Suppressive Study." A chiral assay is in progress. Additional human blood samples (67) were received May 29, 1996 to be assayed for chloroquine concentration.

WR5/P 95-3

We received 111 dog plasma and 12 dose formulation samples on April 26, 1996. The dog plasma samples are being analyzed in accordance to the protocol titled "WR 238605 Multiple Drug Interaction Study in Beagle Dogs."

WR5/P 96-2

Samples are to be analyzed in accordance with the protocol titled "Dose-Ranging Study of the Safety and Efficacy of WR 238605 in the Prevention of Relapse of *Plasmodium vivax* Infection in Thailand." Assay of samples received on September 26, 1996 (266 human blood and 266 human plasma) is in progress.

WR 6026, WR 211,789 and WR 254,421 in Plasma, Blood and Urine

WR6/PU 93-1: Data was presented in the Midterm Report dated August 14, 1994.

Routine Analysis Report WR6/PU 93-01, titled "Routine Analysis for WR 6026 and WR 211,789 in Plasma and WR 6026, WR 211,789 and WR 254,421 in Urine of Samples Obtained under the Protocol Titled 'Phase II Clinical Trial of Oral WR 6026 2 HCl in Patients with Visceral Leishmaniasis - Initial Dose Ranging for Efficacy, Safety and Tolerance,'" will cover results from WR6/P 92-01 and WR6/PU 93-01.

No. of Samples	Description	Date Received	Status
64	plasma (WR6/P 92-01)	3/31/92	Faxed to COR 6/3/92
130	plasma (WR6/P 92-01)	6/3/92	Faxed to COR 6/24/92
117	plasma (WR6/P 93-01)	2/11/93	Data in preparation
68	urine (WR6/P 93-01)	2/11/93	Faxed to COR 3/12/93

WR6/PU 94-3

Samples are for routine analysis in accordance with the protocol titled "Clinical Trial of Oral WR6026•2HCl in Patients with Brazilian Visceral Leishmaniasis due to *L. chagasi*: Initial Dose Range Determination for Efficacy, Safety and Tolerance." Status of samples received is described in the table below.

No. of Samples	Description	Date Received	Status
38 37	human plasma human urine	3/13/96	Preliminary data faxed to COR 7/3/96 Final data faxed to COR 1/27/97
36 17	human plasma human urine	9/24/96	Final data faxed to COR 1/27/97

Gentamicin and Paromomycin

Gnt/p 96-3

Samples are to be analyzed in accordance with the protocol titled " Irritant and Phototoxicity Reactions to the Topical Antileishmanial WR 279396: A Randomized, Double-Blind Phase I Study." Status of samples received is described in the table below.

No. of Samples	Description	Date Received	Status
36	human plasma	8/7/96	Preliminary results faxed to COR 8/26/96 & final results faxed to COR 12/18/96
47	human plasma	10/24/96	Final results faxed to COR 12/18/96

Pyridostigmine

Analysis of 2639 human plasma samples for determination of the concentrations of pyridostigmine cation was accomplished by use of an HPLC method described in Study Report No. 25. The samples were obtained from the South Florida Drug Research Corporation in accordance with the protocol titled "A Study to Evaluate the Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Pyridostigmine when given in Single and Multiple Doses to Males and Females in Different Weight Groups." Analytical results

are presented in the report dated April 3, 1996 for plasma samples from male and female volunteers from analyses performed from December 12, 1994 through May 31, 1995.

CONCLUSIONS

Work on development and/or validation of analytical methodologies during the current contract focused on assays for WR 238,605 (and its stereoisomers), halofantrine (and its metabolite and their stereoisomers), WR 6026 (and its metabolites), mefloquine (and its metabolite), artelinic acid, *p*-aminoheptanophenone (and related compounds), primaquine (and its metabolite), gentamicin and paromomycin, pyridostigmine, WR 242511, chloroquine (and its metabolites), WR 243,251, quinine and doxycycline. Work on routine analyses of biological specimens during this period was performed for studies that required determination of concentrations of WR 238,605 (and its stereoisomers), halofantrine (and its metabolite and their stereoisomers), WR 6026 (and its metabolites), mefloquine (and its metabolite), *p*-aminoheptanophenone (and related compounds), primaquine (and its metabolite), gentamicin and paromomycin, pyridostigmine, chloroquine (and its metabolites), quinine, and doxycycline.

Using the procedures described in this report, we were able to work sequentially or simultaneously on eleven projects (1-WR 238,605, 2-halofantrine (and its metabolite), 3-WR 6026 (and its metabolites), 4-mefloquine (and its metabolite), 5-artelinic acid, 6-*p*-aminoheptanophenone (and related compounds), 7-primaquine (and its metabolite), 8-gentamicin and paromomycin, 9-pyridostigmine, 10-chloroquine (and its metabolites), and 11-a multiple drug interaction study in dog plasma for WR 238,605, mefloquine, chloroquine, quinine, doxycycline, and halofantrine with additional work on development and validation of LC/MS/MS methods for halofantrine (and its metabolite), WR 238,605) in terms of method development, validation, and characterization. We worked on demonstrating sensitivity, specificity, linearity, lack of interferences, accuracy, and reproducibility of the analytical method, describing the extent of recovery for the method, and reporting on the stability of compounds of interest in specimens during storage and drug analysis to provide documentation in support of Investigational New Drug (IND) submissions to the Food and Drug Administration (FDA).

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