

CONTRACT NUMBER DAMD17-92-C-2028

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

FINAL REPORT - APPENDIX A  
for the Period January 15, 1992 - January 14, 1996

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TITLE: Analysis of Investigational Drugs in Biological Fluids-  
Method Development and Routine Assay

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CONTRACTING ORGANIZATION: University of California,  
San Francisco  
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REPORT DATE: February 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command Fort  
Detrick, Frederick, Maryland 21702-5012

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THIS QUALITY INSPECTURE 1

## REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

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| 4. TITLE AND SUBTITLE<br>Analysis of Investigational Drugs in<br>Biological Fluids-Method Development and Routine Assay                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |  |                                                             |  | 5. FUNDING NUMBERS<br>DAMD17-92-C-2028                            |  |
| 6. AUTHOR(S)<br><br>Emil T. Lin, Ph.D.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |  |                                                             |  |                                                                   |  |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>University of California, San Francisco<br>San Francisco, California 94143-0962                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |  |                                                             |  | 8. PERFORMING ORGANIZATION<br>REPORT NUMBER                       |  |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick<br>Frederick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |  |                                                             |  | 10. SPONSORING/MONITORING<br>AGENCY REPORT NUMBER                 |  |
| 11. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |  |                                                             |  |                                                                   |  |
| 12a. DISTRIBUTION/AVAILABILITY STATEMENT<br>Approved for public release; distribution unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |  |                                                             |  | 12b. DISTRIBUTION CODE                                            |  |
| 13. ABSTRACT (Maximum 200 words)<br>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). |  |                                                             |  |                                                                   |  |
| 14. SUBJECT TERMS<br>Investigational Drugs, CD                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |  |                                                             |  | 15. NUMBER OF PAGES<br>503                                        |  |
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| 17. SECURITY CLASSIFICATION<br>OF REPORT<br>Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |  | 18. SECURITY CLASSIFICATION<br>OF THIS PAGE<br>Unclassified |  | 19. SECURITY CLASSIFICATION<br>OF ABSTRACT<br>Unclassified        |  |
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## APPENDIX A

## Method Validation Data

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**LABORATORY METHODOLOGY FOR WR 238,605 AS FREE BASE RAT  
PLASMA ASSAY,\* STUDY REPORT 13, SUPPLEMENT I**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Altex Model 100A Solvent Delivery Module (Beckman Instruments Inc., Berkeley, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. WR 238,605 succinate (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK 73252, expiration date not available.
4. WR 6026 dihydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK01845, expiration date not available.
5. Phosphoric acid (85%) (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
7. Sodium hydroxide (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Methyl *t*-butyl ether (Fisher Scientific, Fair Lawn, NJ).
10. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

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\* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

## C. ASSAY CONDITIONS

## 1. DETECTOR

## Settings

Wavelength: excitation - 375 nm, emission - 480 nm

Sensitivity: high

Range: 4

Response: medium

## Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

## 2. COLUMN

Phenomenex Silica, 5  $\mu$ m particle size, 4.6 x 250 mm  
(Phenomenex Inc., Rancho Palos Verdes, CA).

## 3. SOLVENT SYSTEM

Combine and mix H<sub>2</sub>O (2 L) + (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (20 mL of 1 M  
(NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) + CH<sub>3</sub>CN (2 L).Adjust apparent pH to 7.0 with 85% H<sub>3</sub>PO<sub>4</sub>.

## 4. FLOW RATE

1.2 ml/min

5. STOCK SOLUTIONS - Solutions were stored in a freezer and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

## a. WR 238,605 (free base)

| Preparation date: 8/18/93 |                               |                   |                      |          |                        |
|---------------------------|-------------------------------|-------------------|----------------------|----------|------------------------|
| Solution Type             | Weight of<br>Standard<br>(mg) | Purity<br>Factor* | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>( $\mu$ g/ml) |
| Standard Curve            | 12.56                         | 0.79695           | 50                   | methanol | 200                    |
| Preparation date: 6/21/93 |                               |                   |                      |          |                        |
| Precision                 | 12.79                         | 0.79695           | 50                   | methanol | 204                    |

\* = Molecular weights of WR 238605 free base/WR 238605 succinate

## b. WR 6026 internal standard.

| Preparation date: 6/21/93 |                         |               |                |          |               |
|---------------------------|-------------------------|---------------|----------------|----------|---------------|
| Solution Type             | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
| Internal Std              | 11.16                   | 1             | 100            | methanol | 112           |

## 2. WORKING SOLUTIONS - Store solution in a freezer and discard within 6 months.

## a. WR 238605.

| Preparation date: 8/18/93 |                       |                |          |               |
|---------------------------|-----------------------|----------------|----------|---------------|
| Solution Type             | Conc. Diluted (µg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
| Standard Curve            | 200                   | 1:100          | methanol | 2.00          |
| Standard Curve            | 2.00                  | 1:10           | methanol | 0.200         |

| Preparation date: 7/8/93 |      |       |          |       |
|--------------------------|------|-------|----------|-------|
| Precision                | 204  | 1:100 | methanol | 2.04  |
| Precision                | 2.04 | 1:10  | methanol | 0.204 |

## b. WR 6026 (Internal Standard).

| Preparation date: 6/21/93 |                       |                |          |               |
|---------------------------|-----------------------|----------------|----------|---------------|
| Solution Type             | Conc. Diluted (µg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
| Internal Std.             | 112                   | 1:4            | methanol | 27.9          |

## 7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

a. WR 238,605 as free base - 5.1 min

b. WR 6026 as free base (Internal Standard) - 7.2 min

## 8. BLANK RAT PLASMA

Rat plasma (3.8% sodium citrate) Pel-Freez Biologicals, Rogers, AK.

## 9. INJECTION VOLUME

50-100 µl

## 10. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of  $1/y_i$ .

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum WR 238,605 (as free base) quantitation limit for the assay of rat plasma was based on interday and intraday precision results (Tables 2 and 3) and on standard curve calibrator results (Table 4).)

Approximately 2 ng/ml WR 238,605 (free base) in plasma.

## 12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

Plasma samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

## 13. WISP OPERATING TEMPERATURE

Room temperature.

## D. SAMPLE STORAGE

All samples are to be kept frozen at  $-20^{\circ}\text{C}$  before analysis and thawed at room temperature for preparation (within 30 min) and analysis.

## E. SAMPLE PREPARATION

1. If frozen, thaw rat plasma sample at room temperature and vortex for 1 min. Pipet 0.2 ml of rat plasma into a 13 x 100 glass culture tube.
2. Spike standard curve samples with 00,\* 0,\*\* 1, 2, 4, 8, or 15  $\mu\text{l}$  of 0.200  $\mu\text{g}/\text{ml}$  WR 238605 working solution or 3, 5, 10, 20, or 40  $\mu\text{l}$  of 2.00  $\mu\text{g}/\text{ml}$  WR 238605 working solution to make a standard curve. Since 0.2 ml plasma samples are assayed, this procedure is equivalent to making standard curve samples with WR 238605 concentrations corresponding to 00, 0, 1.00, 2.00, 4.00, 8.00, 15.0, 30.0, 50.0, 100, 200 and 400 ng/ml. Vortex for 20 s.

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\* 00 = Sample with no drug and no internal standard.

\*\* 0 = Sample with no drug but with internal standard.

3. Add 20  $\mu$ l of internal standard working solution (27.9  $\mu$ g/ml WR 6026), except to 00 standard curve sample. Vortex 30 s.
4. Add 0.1 ml of 0.1 M NaOH. Vortex 30 s.
5. Add 3 ml of methyl *t*-butyl ether. Vortex for 1 min, twice, and centrifuge for 10 min at 3000 g.
6. Transfer the organic layer to a second 13 x 100 tube with a pasteur pipette and evaporate to dryness under nitrogen.
7. Reconstitute with 200  $\mu$ l of 50% CH<sub>3</sub>CN, transfer to WISP insert and inject onto column.

#### F. QUALITY CONTROL

1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

2. Pipette Calibration

See ASOP 2C-1.1.

3. Balance Calibration

See ASOP 2C-2.1.

#### G. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the WR 238,605 (as free base) to internal standard peak height ratios in reference samples to the peak height ratios in plasma samples. Plasma (0.2 ml) and reference samples were spiked with corresponding amounts of WR 238,605 (as free base). Each plasma sample was prepared as described in "Sample Preparation" (Section E), except 2.5 ml of the extraction solvent was taken for evaporation (step 6), and the internal standard was added after the evaporation (step 6 not step 3) of the extraction solvent. The plasma samples were generated by spiking 0.2 ml of blank rat plasma with appropriate amounts of drug prior to addition of 0.1 N NaOH buffer. The reference samples (0.2 ml of blank rat plasma) were generated by spiking after the methyl *t*-butyl ether extraction with drug to the 2.5 ml of extraction solvent that was taken.

## H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.2 ml plasma specimens with WR 238,608 (as free base) working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

### Generation of Precision Samples

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>( $\mu$ l) | Precision Sample<br>Nominal Concentration<br>(ng/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------------|------------------------------------------------------|
| X-Lo | 2                              | 0.204                                              | 200                             | 2.04                                                 |
| Low  | 15                             | 0.204                                              | 200                             | 15.3                                                 |
| Med. | 5                              | 2.04                                               | 200                             | 51.0                                                 |
| Hi   | 20                             | 2.04                                               | 200                             | 204                                                  |

## I. RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in a representative standard curve for WR 238,605 as free base appear in Figure 1. Peak height ratios for these calibrators appear in Table 1.

### 2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 2 and 3.

### 3. STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 4

### 4. RECOVERY

Results for this evaluation appear in Table 5.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR  
WR 238,605 (FREE BASE) RAT PLASMA ASSAY,  
STUDY REPORT 13, SUPPLEMENT NO. I

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |        | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|--------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) | HEIGHT |                           |                                        |
| 0                         | 0                        | 0      | -                         | -                                      |
| 0.200                     | 1.00                     | 0.034  | 1.13                      |                                        |
| 0.400                     | 2.00                     | 0.065  | 2.15                      |                                        |
| 0.801                     | 4.00                     | 0.116  | 3.81                      |                                        |
| 1.60                      | 8.00                     | 0.230  | 7.53                      |                                        |
| 3.00                      | 15.0                     | 0.483  | 15.8                      |                                        |
| 6.01                      | 30.0                     | 0.849  | 27.7                      |                                        |
| 10.0                      | 50.0                     | 1.457  | 47.6                      |                                        |
| 20.0                      | 100                      | 2.944  | 96.1                      |                                        |
| 40.0                      | 200                      | 6.413  | 209                       |                                        |
| 80.1                      | 400                      | 12.242 | 400                       |                                        |

Regression equation:

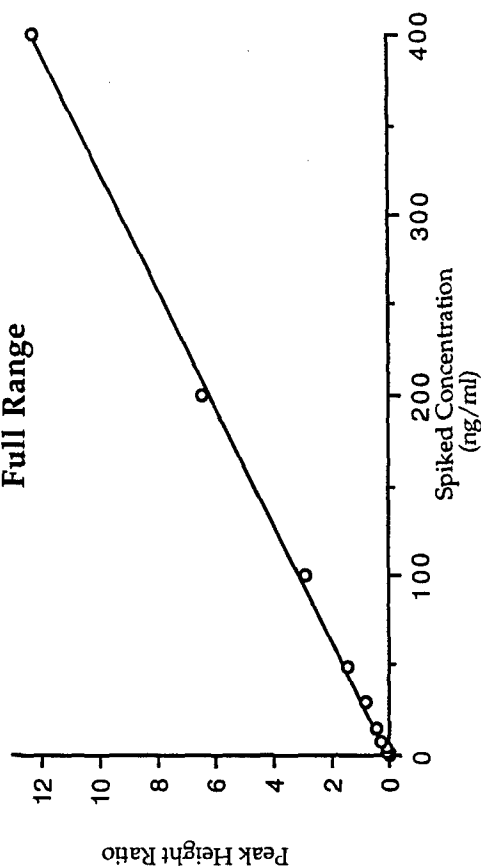
$$y = 0.03060x - 0.00074, \quad r^2 = 0.9987$$

\* Into 0.2 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

Representative Standard Curve  
Full Range



Expanded View of Near Zero Range

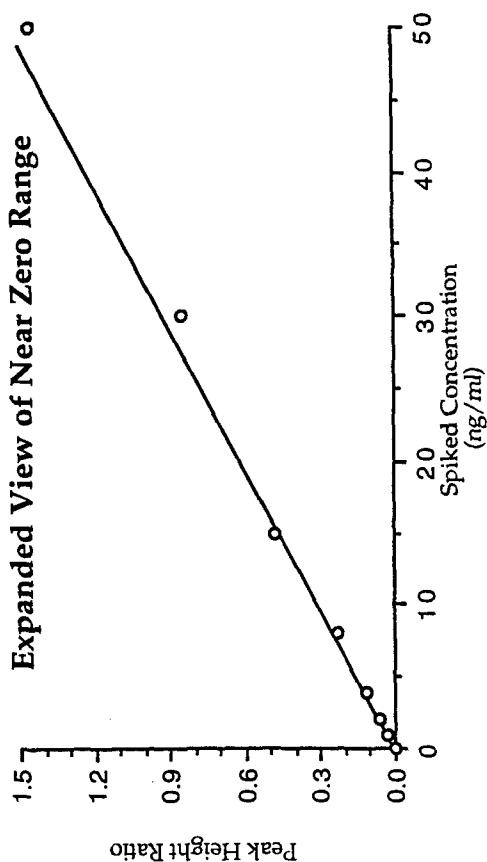


**TABLE 2: INTERDAY PRECISION OF WR 238,605 FREE BASE RAT PLASMA ASSAY**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER |      |                                          | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|---------------|------|------------------------------------------|-----------------|-----------------|-----------------|------------------|
|                            | 1             | 2    | 3<br>Measured Concentrations*<br>(ng/ml) |                 |                 |                 |                  |
| 2.04                       | 2.13          | 2.09 | 1.94                                     | 2.05            | 0.100           | 4.88            | 0.654            |
| 15.3                       | 14.8          | 14.9 | 16.4                                     | 15.4            | 0.896           | 5.83            | 0.436            |
| 51.0                       | 54.6          | 45.9 | 55.5                                     | 52.0            | 5.30            | 10.2            | 1.96             |
| 204                        | 230           | 218  | 239                                      | 229             | 10.5            | 4.60            | 12.3             |

**TABLE 3: INTRADAY PRECISION OF WR 238,605 FREE BASE RAT PLASMA ASSAY**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER |      |      |      |      |                                         | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|---------------|------|------|------|------|-----------------------------------------|-----------------|-----------------|-----------------|------------------|
|                            | 1             | 2    | 3    | 4    | 5    | 6<br>Measured Concentrations<br>(ng/ml) |                 |                 |                 |                  |
| 2.04                       | 2.35          | 2.07 | 1.86 | 2.21 | 1.75 | 2.03                                    | 2.05            | 0.220           | 10.8            | 0.245            |
| 15.3                       | 15.0          | 14.9 | 14.6 | 14.3 | 14.8 | 15.1                                    | 14.8            | 0.293           | 1.98            | -3.38            |
| 51.0                       | 43.4          | 44.2 | 46.8 | 46.6 | 42.3 | 46.8                                    | 45.0            | 1.98            | 4.39            | -11.7            |
| 204                        | 215           | 210  | 212  | 216  | 216  | 216                                     | 214             | 2.56            | 1.20            | 4.98             |

\* Measured concentrations are averages of two analyses.

**TABLE 4: STUDY STANDARD CURVE CALIBRATOR STATISTICAL  
PARAMETERS FOR WR 238,605 RAT PLASMA ASSAY,  
STUDY REPORT 13 SUPPLEMENT I**

| Spiked<br>Concentration<br>(ng/ml) | n | Mean<br>(ng/ml) | Standard<br>Deviation<br>(ng/ml) | Percent<br>C.V. | Percent<br>Deviation |
|------------------------------------|---|-----------------|----------------------------------|-----------------|----------------------|
| 1.00                               | 3 | 1.25            | 0.188                            | 15.0            | 25.3                 |
| 2.00                               | 3 | 2.05            | 0.111                            | 5.40            | 2.33                 |
| 4.00                               | 3 | 3.89            | 0.067                            | 1.71            | -2.83                |
| 8.00                               | 3 | 7.75            | 0.276                            | 3.56            | -3.13                |
| 15.0                               | 3 | 15.4            | 0.551                            | 3.57            | 2.89                 |
| 30.0                               | 3 | 27.5            | 0.473                            | 1.72            | -8.22                |
| 50.0                               | 3 | 46.0            | 2.86                             | 6.21            | -8.00                |
| 100                                | 3 | 93.2            | 2.51                             | 2.70            | -6.80                |
| 200                                | 3 | 210             | 1.73                             | 0.82            | 5.00                 |
| 400                                | 3 | 405             | 6.81                             | 1.679           | 1.33                 |

TABLE 5: RECOVERY OF WR 238,605 FROM RAT PLASMA

| SAMPLE<br>ID                 | SPIKED<br>CONCENTRATION<br>Range (ng/ml) |      | PEAK HEIGHT RATIO |               | MEAN<br>PERCENT<br>RECOVERY |
|------------------------------|------------------------------------------|------|-------------------|---------------|-----------------------------|
|                              |                                          |      | REFERENCE         | PLASMA        |                             |
| <u>WR 238,605</u>            |                                          |      |                   |               |                             |
| 1                            | X Low                                    | 5.00 | 0.087             | 0.052         | 63.9                        |
| 2                            |                                          |      | 0.075             | 0.048         |                             |
| 3                            |                                          |      | 0.071             | 0.049         |                             |
| Mean (± SD)                  |                                          |      | 0.078 ± 0.008     | 0.050 ± 0.002 |                             |
| 1                            | Low                                      | 15.0 | 0.535             | 0.379         | 69.5                        |
| 2                            |                                          |      | 0.469             | 0.336         |                             |
| 3                            |                                          |      | 0.507             | 0.335         |                             |
| Mean (± SD)                  |                                          |      | 0.504 ± 0.033     | 0.350 ± 0.025 |                             |
| 1                            | Medium                                   | 50.0 | 1.774             | 1.183         | 68.7                        |
| 2                            |                                          |      | 1.772             | 1.123         |                             |
| 3                            |                                          |      | 1.698             | 1.299         |                             |
| Mean (± SD)                  |                                          |      | 1.748 ± 0.043     | 1.202 ± 0.089 |                             |
| 1                            | High                                     | 200  | 7.513             | 5.135         | 63.5                        |
| 2                            |                                          |      | 7.421             | 4.799         |                             |
| 3                            |                                          |      | 7.932             | 4.577         |                             |
| Mean (± SD)                  |                                          |      | 7.622 ± 0.272     | 4.837 ± 0.281 |                             |
| AVERAGE MEAN RECOVERY = 66.4 |                                          |      |                   |               |                             |

TABLE 5: RECOVERY OF WR 238,605 FREE BASE FROM DOG PLASMA

| SAMPLE<br>ID                         | SPIKED<br>CONCENTRATION<br>Range (ng/ml) |      | PEAK HEIGHT RATIO |               | MEAN<br>PERCENT<br>RECOVERY |
|--------------------------------------|------------------------------------------|------|-------------------|---------------|-----------------------------|
|                                      |                                          |      | REFERENCE         | PLASMA        |                             |
| <u>WR 238,605 free base</u>          |                                          |      |                   |               |                             |
| 1                                    | X Low                                    | 5.00 | 0.069             | 0.048         | 70.4                        |
| 2                                    |                                          |      | 0.066             | 0.045         |                             |
| 3                                    |                                          |      | 0.068             | 0.050         |                             |
| Mean (± SD)                          |                                          |      | 0.068 ± 0.002     | 0.048 ± 0.003 |                             |
| 1                                    | Low                                      | 15.0 | 0.515             | 0.371         | 72.7                        |
| 2                                    |                                          |      | 0.505             | 0.366         |                             |
| 3                                    |                                          |      | 0.484             | 0.356         |                             |
| Mean (± SD)                          |                                          |      | 0.501 ± 0.016     | 0.364 ± 0.008 |                             |
| 1                                    | Medium                                   | 50.0 | 1.614             | 1.105         | 63.3                        |
| 2                                    |                                          |      | 1.721             | 1.008         |                             |
| 3                                    |                                          |      | 1.566             | 0.989         |                             |
| Mean (± SD)                          |                                          |      | 1.634 ± 0.079     | 1.034 ± 0.062 |                             |
| 1                                    | High                                     | 200  | 7.368             | 4.342         | 59.7                        |
| 2                                    |                                          |      | 8.223             | 4.882         |                             |
| 3                                    |                                          |      | BC                | 4.749         |                             |
| Mean (± SD)                          |                                          |      | 7.796 ± 0.605     | 4.658 ± 0.281 |                             |
| AVERAGE MEAN PERCENT RECOVERY = 66.5 |                                          |      |                   |               |                             |

**LABORATORY METHODOLOGY FOR WR 238,605 AS FREE BASE  
DOG PLASMA ASSAY,\* STUDY REPORT 13, SUPPLEMENT II**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Altex Model 100A Solvent Delivery Module (Beckman Instruments Inc., Berkeley, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. WR 238,605 succinate (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK 73252, expiration date not available.
4. WR 6026 dihydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK01845, expiration date not available.
5. Phosphoric acid (85%) (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
7. Sodium hydroxide (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Methyl *t*-butyl ether (Fisher Scientific, Fair Lawn, NJ).
10. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

**C. ASSAY CONDITIONS**

---

\* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

## 1. DETECTOR

## Settings

Wavelength: excitation - 375 nm, emission - 480 nm

Sensitivity: high

Range: 4

Response: medium

## Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

## 2. COLUMN

Phenomenex Silica, 5  $\mu$ m particle size, 4.6 x 250 mm  
(Phenomenex Inc., Rancho Palos Verdes, CA).

## 3. SOLVENT SYSTEM

CH<sub>3</sub>CN/H<sub>2</sub>O (50:50, v/v) and 5 mM (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, pH = 7.0  
(adjusted with 85% H<sub>3</sub>PO<sub>4</sub>).

## 4. FLOW RATE

1.2 ml/min

## 5. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

## a. WR 238,605 (free base)

| Preparation date: 1/6/94  |                               |                   |                      |          |                        |
|---------------------------|-------------------------------|-------------------|----------------------|----------|------------------------|
| Solution Type             | Weight of<br>Standard<br>(mg) | Purity<br>Factor* | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>( $\mu$ g/ml) |
| Standard Curve            | 12.56                         | 0.79695           | 50                   | methanol | 200                    |
| Preparation date: 6/21/93 |                               |                   |                      |          |                        |
| Precision                 | 12.79                         | 0.79695           | 50                   | methanol | 204                    |

\* = Molecular weights of WR 238605 free base/WR 238605 succinate

## b. WR 6026 internal standard.

| Preparation date: 1/6/94 |                         |               |                |          |               |
|--------------------------|-------------------------|---------------|----------------|----------|---------------|
| Solution Type            | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
| Internal Std             | 11.16                   | 1             | 100            | methanol | 112           |

## 2. WORKING SOLUTIONS - Store solution at 4°C and discard within 6 months.

## a. WR 238605.

| Preparation date: 1/6/94 |                       |                |          |               |
|--------------------------|-----------------------|----------------|----------|---------------|
| Solution Type            | Conc. Diluted (µg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
| Standard Curve           | 200                   | 1:100          | methanol | 2.00          |
| Precision                | 204                   | 1:100          | methanol | 2.04          |

| Preparation date: day of use |      |      |          |       |
|------------------------------|------|------|----------|-------|
| Standard Curve               | 2.00 | 1:10 | methanol | 0.200 |
| Precision                    | 2.04 | 1:10 | methanol | 0.204 |

## b. WR 6026 (Internal Standard).

| Preparation date: 6/21/93 |                       |                |          |               |
|---------------------------|-----------------------|----------------|----------|---------------|
| Solution Type             | Conc. Diluted (µg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
| Internal Std.             | 112                   | 1:4            | methanol | 27.9          |

## 7. RETENTION TIMES (subject to change depending on temperature and column performance).

a. WR 238,605 as free base - 5.1 min

b. WR 6026 as free base (Internal Standard) - 7.2 min

## 8. BLANK DOG PLASMA

Dog plasma (3.8% sodium citrate, heparin) Pel-Freez Biologicals, Rogers, AK.

## 9. INJECTION VOLUME

50-100 µl

#### 10. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression.

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum quantitation limit was determined as the WR 238,605 (free base) standard curve concentration at which the signal to noise ratio was at least 3 to 1.)

1.00 ng/ml WR 238,605 (free base) in plasma.

#### 12. SAMPLE VOLUME MEASUREMENT

Plasma sample volumes were measured with a variable volume Eppendorf pipetter.

#### 13. WISP OPERATING TEMPERATURE

Room temperature.

### D. SAMPLE STORAGE

All samples are to be kept frozen at -20°C before analysis and thawed at room temperature for preparation (within 30 min) and analysis.

### E. SAMPLE PREPARATION

1. Pipet 0.2 ml of dog plasma into a 13 x 100 culture tube.
2. Spike standard curve samples with 00, 0, 1, 2, 4, 8, or 15 µl of 0.200 µg/ml WR 238605 working solution or 3, 5, 10, 20, or 40 µl of 2.00 µg/ml WR 238605 working solution to make a standard curve. Since 0.2 ml plasma samples are assayed, this procedure is equivalent to making standard curve samples with WR 238605 concentrations corresponding to 00, 0, 1.00, 2.00, 4.00, 8.00, 15.0, 30.0, 50.0, 100, 200 and 400 ng/ml. Vortex for 20 s.
3. Thaw clinical samples at room temperature.
4. Vortex for 1 min.
5. Add 20 µl of internal standard working solution (27.9 µg/ml WR 6026). Vortex 30 s.
6. Add add 0.1 ml of 0.1 M NaOH. Vortex 30 s.

7. Add 3 ml of methyl *t*-butyl ether. Vortex for 2 min and centrifuge for 10 min at 3000 *g*.
8. Transfer the organic layer to a second 13 x 100 tube and evaporate to dryness under nitrogen.
9. Reconstitute with 200  $\mu$ l of 50% CH<sub>3</sub>CN and inject onto column.

#### F. QUALITY CONTROL

1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

2. Pipette Calibration

See ASOP 2C-1.1.

3. Balance Calibration

See ASOP 2C-2.1.

#### G. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the WR 238,605 (as free base) to internal standard peak height ratios in a reference sample to the peak height ratios in plasma. Plasma (0.2 ml) and reference samples were spiked with corresponding amounts of WR 238,605 (as free base). Each plasma sample was prepared as described in "Sample Preparation" (Section E), except samples were centrifuged for 10 minutes (step 7), 2.5 ml of sample was taken for evaporation (step 8), and the internal standard was added after the evaporation (step 8). The reference samples were spiked prior to extraction (step 5) with drug and after evaporation (step 8) with internal standard.

#### H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.2 ml plasma specimens with WR 238,608 (as free base) working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

## Generation of Precision Samples

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>( $\mu$ l) | Precision Sample<br>Nominal Concentration<br>(ng/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------------|------------------------------------------------------|
| X-Lo | 2                              | 0.204                                              | 200                             | 2.04                                                 |
| Low  | 15                             | 0.204                                              | 200                             | 15.3                                                 |
| Med. | 5                              | 2.04                                               | 200                             | 51.0                                                 |
| Hi   | 20                             | 2.04                                               | 200                             | 204                                                  |

## I. RESULTS

## 1. STANDARD CURVE

Chromatograms for each point in a representative standard curve for WR 238,605 as free base appear in Figure 1. Peak height ratios for these calibrators appear in Table 1.

## 2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 2 and 3.

## 3. STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 4

## 4. RECOVERY

Results for this evaluation appear in Table 5.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR  
WR 238,605 (FREE BASE) DOG PLASMA ASSAY,  
STUDY REPORT 13, SUPPLEMENT II

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |        | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|--------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) |        |                           |                                        |
| 0                         | 0                        | 0      | -                         | -                                      |
| 0.200                     | 1.00                     | 0.031  | 1.13                      |                                        |
| 0.400                     | 2.00                     | 0.061  | 2.15                      |                                        |
| 0.800                     | 4.00                     | 0.107  | 3.72                      |                                        |
| 1.60                      | 8.00                     | 0.219  | 7.55                      |                                        |
| 3.00                      | 15.0                     | 0.466  | 16.0                      |                                        |
| 6.01                      | 30.0                     | 0.826  | 28.3                      |                                        |
| 10.0                      | 50.0                     | 1.527  | 52.2                      |                                        |
| 20.0                      | 100                      | 2.501  | 85.5                      |                                        |
| 40.0                      | 200                      | 6.253  | 214                       |                                        |
| 80.0                      | 400                      | 11.817 | 404                       |                                        |

Regression equation:\*\*\*  
 $y = 0.0293x - 0.00198$ ,  $r^2 = 0.9951$

\* Into 0.2 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y$ .

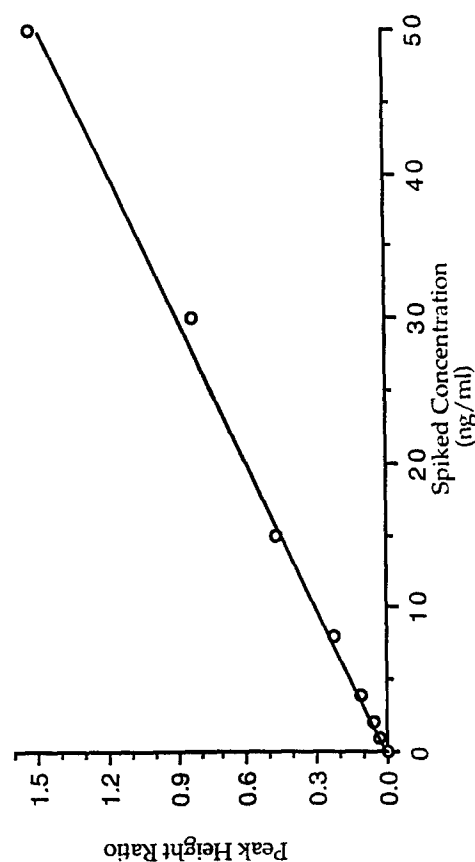
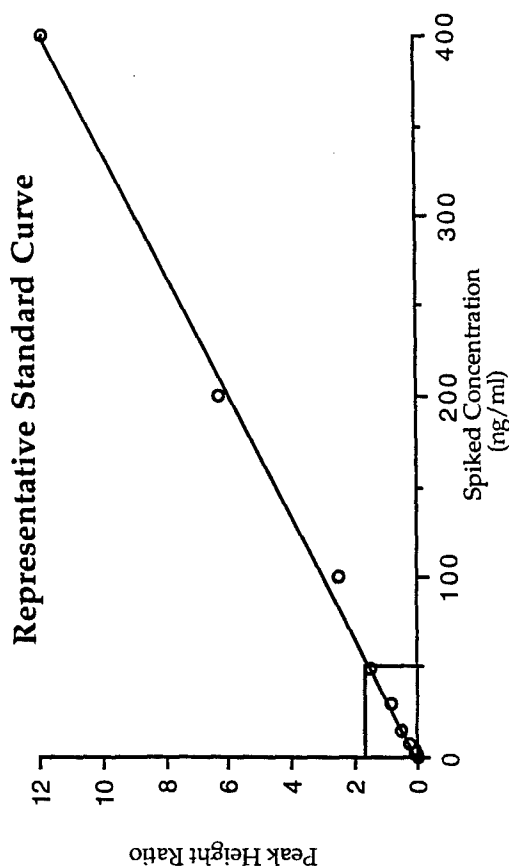


**TABLE 2: INTERDAY PRECISION OF WR 238,605 FREE BASE DOG PLASMA ASSAY**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER |      |                                          | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|---------------|------|------------------------------------------|-----------------|-----------------|-----------------|------------------|
|                            | 1             | 2    | 3<br>Measured Concentrations*<br>(ng/ml) |                 |                 |                 |                  |
| 2.04                       | 2.44          | 2.43 | 2.38                                     | 2.41            | 0.033           | 1.38            | 18.3             |
| 15.3                       | 14.3          | 14.1 | 14.4                                     | 14.3            | 0.076           | 0.54            | -6.75            |
| 51.0                       | 54.4          | 51.5 | 59.0                                     | 55.1            | 3.56            | 6.46            | 8.07             |
| 204                        | 222           | 208  | 221                                      | 218             | 6.54            | 3.01            | 6.62             |

**TABLE 3: INTRADAY PRECISION OF WR 238,605 FREE BASE DOG PLASMA ASSAY**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER |      |      |      |      |                                         | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|---------------|------|------|------|------|-----------------------------------------|-----------------|-----------------|-----------------|------------------|
|                            | 1             | 2    | 3    | 4    | 5    | 6<br>Measured Concentrations<br>(ng/ml) |                 |                 |                 |                  |
| 2.04                       | 2.34          | 2.04 | 2.21 | 2.01 | 1.91 | 2.14                                    | 2.11            | 0.154           | 7.31            | 3.35             |
| 15.3                       | 14.9          | 14.4 | 13.7 | 16   | 13.1 | 14.6                                    | 14.5            | 1.00            | 6.93            | -5.56            |
| 51.0                       | 50.4          | 60.7 | 46.7 | 51.9 | 47.1 | 49.1                                    | 51.0            | 5.15            | 10.1            | -0.033           |
| 204                        | 217           | 200  | 213  | 201  | 192  | 199                                     | 204             | 9.42            | 4.62            | -0.163           |

\* Measured concentrations are averages of two analyses.

**TABLE 4: STUDY STANDARD CURVE CALIBRATOR STATISTICAL  
PARAMETERS FOR WR 238,605 DOG PLASMA ASSAY,  
STUDY REPORT 13 SUPPLEMENT**

| Spiked<br>Concentration<br>(ng/ml) | n | Mean<br>(ng/ml) | Standard<br>Deviation<br>(ng/ml) | Percent<br>C.V. | Percent<br>Deviation |
|------------------------------------|---|-----------------|----------------------------------|-----------------|----------------------|
| 1.00                               | 4 | 1.22            | 0.0818                           | 6.72            | 21.8                 |
| 2.00                               | 4 | 2.09            | 0.0920                           | 4.40            | 4.50                 |
| 4.00                               | 4 | 3.73            | 0.135                            | 3.62            | -6.81                |
| 8.00                               | 4 | 7.47            | 0.199                            | 2.66            | -6.59                |
| 15.0                               | 4 | 14.4            | 1.11                             | 7.75            | -4.33                |
| 30.0                               | 4 | 27.5            | 1.35                             | 4.93            | -8.50                |
| 50.0                               | 4 | 52.1            | 0.926                            | 1.78            | 4.10                 |
| 100                                | 4 | 97.8            | 8.39                             | 8.58            | -2.18                |
| 200                                | 4 | 211             | 6.95                             | 3.30            | 5.38                 |
| 400                                | 4 | 396             | 9.26                             | 2.34            | -1.13                |

TABLE 5: RECOVERY OF WR 238,605 FREE BASE FROM DOG PLASMA

| SAMPLE ID                            | SPIKED CONCENTRATION<br>Range (ng/ml) |      | PEAK HEIGHT RATIO |               | MEAN PERCENT RECOVERY |
|--------------------------------------|---------------------------------------|------|-------------------|---------------|-----------------------|
|                                      |                                       |      | REFERENCE         | PLASMA        |                       |
| WR 238,605 free base                 |                                       |      |                   |               |                       |
| 1                                    | X Low                                 | 5.00 | 0.069             | 0.048         | 70.4                  |
| 2                                    |                                       |      | 0.066             | 0.045         |                       |
| 3                                    |                                       |      | 0.068             | 0.050         |                       |
| Mean (± SD)                          |                                       |      | 0.068 ± 0.002     | 0.048 ± 0.003 |                       |
| 1                                    | Low                                   | 15.0 | 0.515             | 0.371         | 72.7                  |
| 2                                    |                                       |      | 0.505             | 0.366         |                       |
| 3                                    |                                       |      | 0.484             | 0.356         |                       |
| Mean (± SD)                          |                                       |      | 0.501 ± 0.016     | 0.364 ± 0.008 |                       |
| 1                                    | Medium                                | 50.0 | 1.614             | 1.105         | 63.3                  |
| 2                                    |                                       |      | 1.721             | 1.008         |                       |
| 3                                    |                                       |      | 1.566             | 0.989         |                       |
| Mean (± SD)                          |                                       |      | 1.634 ± 0.079     | 1.034 ± 0.062 |                       |
| 1                                    | High                                  | 200  | 7.368             | 4.342         | 59.7                  |
| 2                                    |                                       |      | 8.223             | 4.882         |                       |
| 3                                    |                                       |      | BC                | 4.749         |                       |
| Mean (± SD)                          |                                       |      | 7.796 ± 0.605     | 4.658 ± 0.281 |                       |
| AVERAGE MEAN PERCENT RECOVERY = 66.5 |                                       |      |                   |               |                       |

**LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460  
(AS FREE BASES) BLOOD AND PLASMA ASSAY,\* STUDY REPORT 17**

**presented in mid term report**

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\* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

**LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460  
(AS FREE BASES) BLOOD AND PLASMA ASSAY,\* STUDY REPORT 17B**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. LC-600 Shimadzu Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade unless otherwise specified.
2. All chemicals are reagent grade unless otherwise specified.
3. Halofantrine hydrochloride, WR 171,669, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.).
3. WR 178,460, bottle no. BK 21070 (Walter Reed Army Institute of Research, Washington D.C.).
4. WR 122,455 , bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.).
5. Methanol Optima Grade (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile (Fisher Scientific, Fair Lawn, NJ).
7. Methyl *t* butyl ether (Baxter, Burdick & Jackson, Muskegon, MI).
8. Sodium hydroxide (Mallinckrodt Co., Paris, KY).
9. Water (deionized by Nanopure II, Barnstead Co., Boston, MA).
10. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

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\* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

### C. ASSAY CONDITIONS

#### 1. DETECTOR

##### Settings

Wavelength; excitation - 300 nm, emission - 375 nm

Sensitivity - high and Range - 32

Lamp: Ushio xenon, type UXL-155-LCA(S-LC)

#### 2. COLUMN

Phenomenex Silica, 5  $\mu$ m particle size, 4.6 x 250 mm

(Phenomenex Inc., Rancho Palos Verdes, CA) or equivalent.

#### 3. SOLVENT SYSTEM

CH<sub>3</sub>OH/H<sub>2</sub>O (80:20, v/v) + 5 mM (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (final concentration)  
(Optima methanol)

#### 4. FLOW RATE

1.0 ml/min

#### 5. REPRESENTATIVE STOCK SOLUTIONS - Stock solutions of halofantrine, its metabolite and the internal standard were kept at -20°C and covered in aluminum foil. Stock solutions of halofantrine and its metabolite were checked for deterioration by HPLC comparison of neat injections to newly made solutions (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 6 months after the preparation date).

##### a. Halofantrine (free base) for interday plasma precision.

Prep date: 9/7/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------------|
| Standard Curve | 5.54                    | 0.942          | 50             | methanol | 104                 |
| Control        | 5.44                    | 0.942          | 50.6           | methanol | 101                 |

\*= Molecular weights of halofantrine free base/halofantrine hydrochloride

##### b. WR 178,460 (free base) for interday plasma precision.

Prep date: 9/7/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------------|
| Standard Curve | 5.62                    | 0.924          | 50             | methanol | 104                 |
| Control        | 5.62                    | 0.924          | 50             | methanol | 104                 |

\*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

## c. WR 122,455 - Internal standard for interday plasma precision.

Prep date: 7/13/94

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|---------------|-------------------------|---------------|----------------|----------|---------------|
| Internal std. | 5.10                    | 1             | 25             | methanol | 204           |

## 6. REPRESENTATIVE WORKING SOLUTIONS - Solutions were stored in a -20°C freezer, covered in aluminum foil, and discarded when stock solutions were discarded or by 6 months after the preparation date).

## a. Mixed halofantrine and WR 178,460 (as free bases) solutions.

Low concentration solution. Combine 0.500 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 0.500               | 100            | methanol | 0.520         |
| Standard Curve (WR 178,460)   | 104                   | 0.500               | 100            | methanol | 0.520         |

High concentration solution. Combine 2.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 2.00                | 100            | methanol | 2.08          |
| Standard Curve (WR 178,460)   | 104                   | 2.00                | 100            | methanol | 2.08          |

Low concentration solution. Combine 0.500 ml each of halofantrine and WR 178,460 (as free bases) stock control curve solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

| Solution Type          | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Control (Halofantrine) | 101                   | 0.500               | 100            | methanol | 0.505         |
| Control (WR 178,460)   | 104                   | 0.500               | 100            | methanol | 0.520         |

High concentration solution. Combine 2.00 ml each of halofantrine and WR 178,460 (as free bases) stock control solutions and q.s. to 100 ml for interday plasma precision.

Prep date: 9/13/94

| Solution Type             | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>(ng/ml) |
|---------------------------|-------------------------------------------------|---------------------------|----------------------|----------|------------------|
| Control<br>(Halofantrine) | 101                                             | 2.00                      | 100                  | methanol | 2.02             |
| Control<br>(WR 178,460)   | 104                                             | 2.00                      | 100                  | methanol | 2.08             |

- b. WR 122,455 - Internal standard for interday plasma precision.

Prep date: 9/7/94

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Volume<br>Diluted<br>(part) | QS<br>Volume<br>(part) | Solvent  | Conc.<br>( $\mu\text{g}/\text{ml}$ ) |
|---------------|-------------------------------------------------|-----------------------------|------------------------|----------|--------------------------------------|
| Internal std. | 204                                             | 0.5                         | 200                    | methanol | 0.510                                |

7. RETENTION TIMES (subject to change depending on temperature and column performance).

- Halofantrine (free base) - 8 min
- WR 178,460 (free base) - 11 min
- WR 122,455 (Internal Standard) - 14 min

8. BLANK PLASMA AND BLOOD

Human plasma and blood (CPD or CPDA-1 as anticoagulant) is obtained from the San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME: Samples that are expected to have high halofantrine or WR 178,460 concentrations (i.e. high standard curve calibrators, high concentration control samples, and sponsor samples shown or expected to be near  $C_{\text{peak}}$ ) are injected at the low end of the volume range.

50-150  $\mu\text{l}$

10. QUANTITATION

By peak height ratio of drug peak and metabolite peak relative to internal standard peak. Standard curves are calculated by non weighted linear regression and are split into low and high range curves.

11. MINIMUM QUANTITATION LIMITS OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the

assay of human and plasma were based on the interday and intraday low point validation results and on standard curve calibrator results.)

2.08 ng/ml halofantrine (free base) in plasma.

2.08 ng/ml WR 178,460 (free base) in plasma.

1.02 ng/ml halofantrine (free base) in blood.

0.964 ng/ml WR 178,460 (free base) in blood.

## 12. SAMPLE VOLUME MEASUREMENT

Plasma sample volumes were measured with a 200  $\mu$ l or a 1000  $\mu$ l Gilson Pipetman. Blood sample volumes were measured with Eppendorf pipettes.

## 13. WISP OPERATING TEMPERATURE

Room temperature.

## 14. SAMPLE EVAPORATION

Extracted samples are evaporated in a N-EVAP® Model 112 (Organomatic Assoc, Inc., S. Berlin, MA) by passing N<sub>2</sub> over the sample. The samples do not sit in water during evaporation.

## D. SAMPLE STORAGE

All samples were kept frozen at -70°C before analysis and thawed and held on ice until prepared for analysis, unless specified otherwise.

## E. SAMPLE PREPARATION

### PLASMA SAMPLES

1. Pipet 0.5 ml of plasma into a 13x100 mm silanized tube on ice.
2. Spike standard curve samples on ice as shown in Section G "Generation of Standard Curve Calibrators" and vortex for 30 s.
3. Add 150  $\mu$ l of internal standard working solution (WR 122,455, 0.51  $\mu$ g/ml) on ice. Vortex for 30 s.
4. Add 1 ml acetonitrile. Vortex for 1 min and repeat. Centrifuge for 10 min at 3000 g.
5. Transfer supernatant to 16x125 mm silanized tube and evaporate to 0.5 ml.
6. Add 0.5 ml water and 50  $\mu$ l of 0.1 N NaOH. Vortex for 30 s.

7. Add 5 ml methyl-*t*-butyl ether. Vortex for 1 min and repeat. Centrifuge for 10 min at 3000 g.
8. Freeze mixture in dry ice/methanol bath. Transfer organic phase to silanized 13x100 mm tube and begin evaporation.
9. Repeat steps 7 and 8, transferring organic phase to same 13x100 mm tube and evaporate to dryness.
10. Reconstitute residue with 200 µl of 80% methanol containing 0.001% HCl. Vortex for 2 min.
11. Transfer to silanized glass WISP inserts and inject onto column.

#### BLOOD SAMPLES

1. Pipet 0.5 ml of blood into a 13x100 mm silanized tube on ice.
2. Follow step 2 as in plasma sample preparation. Vortex for 20 s, and let stand on ice for 1 h.
3. Add (while samples are on ice) 0.5 ml water. Vortex for 10 s.
4. Sonicate for 10 min in water bath.
5. Add 50 µl of internal standard working solution (WR 122,455, 0.51 µg/ml). Vortex for 20 s.
6. Add 2 ml acetonitrile. Vortex for 2.5 min. Centrifuge for 15 min at 3000 g.
7. Follow steps 5-11 as in plasma sample preparation.

#### F. QUALITY CONTROL

##### 1. CONTENT AND FREQUENCY OF BLANKS

A blank plasma or blood sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

##### 2. PIPETTE CALIBRATION

See SOP 2C-1.1.

##### 3. BALANCE CALIBRATION

See SOP 2C-2.1

#### G. GENERATION OF STANDARD CURVE CALIBRATORS

A representative example of the generation of standard curve calibrators is shown in the table below. Spike blank plasma standard curve samples on ice with halofantrine and WR 178,460 (as free bases)

mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 0.500 ml plasma samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

Generation of Halofantrine and WR 178,460 (as free bases)  
Standard Curve Samples

| Sample | Volume Spiked (μl) | Spiking Solution Concentration (μg/ml) | Mass Spiked (ng) | Standard Curve Sample Nominal Concentration (ng/ml) |
|--------|--------------------|----------------------------------------|------------------|-----------------------------------------------------|
| 00*    | 0                  | 0                                      | 0                | 0                                                   |
| 0**    | 0                  | 0                                      | 0                | 0                                                   |
| 1      | 2                  | 0.520                                  | 1.04             | 2.08                                                |
| 2      | 4                  | 0.520                                  | 2.08             | 4.16                                                |
| 3      | 8                  | 0.520                                  | 4.16             | 8.32                                                |
| 4      | 4                  | 2.08                                   | 8.32             | 16.6                                                |
| 5      | 8                  | 2.08                                   | 16.64            | 33.3                                                |
| 6      | 16                 | 2.08                                   | 33.28            | 66.6                                                |
| 7      | 32                 | 2.08                                   | 66.56            | 133                                                 |
| 8      | 64                 | 2.08                                   | 133.12           | 266                                                 |

## H. GENERATION OF PRECISION SAMPLES

A representative example of the generation of precision controls is shown in the table below. Samples for precision analysis were prepared by spiking 0.5 ml plasma or blood specimens with control working solutions to make the halofantrine and WR 178,460 (as free bases) concentrations shown.

Generation of Halofantrine Precision Samples

|      | Volume Spiked (μl) | Spiking Solution Concentration (μg/ml) | Control Volume (ml) | Precision Sample Nominal Concentration (ng/ml) |
|------|--------------------|----------------------------------------|---------------------|------------------------------------------------|
| X-Lo | 4                  | 0.505                                  | 0.5                 | 4.04                                           |
| Low  | 10                 | 0.505                                  | 0.5                 | 10.1                                           |
| Med. | 10                 | 2.02                                   | 0.5                 | 40.4                                           |
| Hi   | 32                 | 2.02                                   | 0.5                 | 129                                            |

Generation of WR 178,460 Precision Samples

|      | Volume Spiked (μl) | Spiking Solution Concentration (μg/ml) | Control Volume (ml) | Precision Sample Nominal Concentration (ng/ml) |
|------|--------------------|----------------------------------------|---------------------|------------------------------------------------|
| X-Lo | 4                  | 0.520                                  | 0.5                 | 4.16                                           |
| Low  | 10                 | 0.520                                  | 0.5                 | 10.4                                           |
| Med. | 10                 | 2.08                                   | 0.5                 | 41.6                                           |
| Hi   | 32                 | 2.08                                   | 0.5                 | 133                                            |

\* 00 = Sample with no drug and no internal standard.

\*\* 0 = Sample with no drug but with internal standard.

## I. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in plasma or blood. Plasma or blood (0.5 ml) samples were spiked with halofantrine and WR 178,460 (as free bases) then prepared as described above in "Sample Preparation," except the internal standard was added after the extracts were evaporated (step 10). Reference samples were generated by spiking reconstitution solvent with drug and internal standard.

## J. GENERATION OF STABILITY SAMPLES

System stability samples were generated in the same way as precision control samples.

Bench top stability samples were generated in the same way as precision control samples at low and high concentrations.

The effect of repeated freeze and thaw cycles on stabilities of halofantrine and WR 178,460 (as free bases) in human plasma and blood samples was determined as follows: Spiked (low and high concentrations) pooled biological sample were subjected to five thaw/freeze cycles. For each cycle, a duplicate set of thaw/freeze samples (0.5 ml) was generated at each concentration. The study is run with the following procedure:

- a. Prepare high and low concentration samples labeled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.
- b. Store all samples until frozen at the specified temperature.
- c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 h.

| Cycle | Keep these<br>samples in freezer | Thaw these<br>samples |
|-------|----------------------------------|-----------------------|
| 1     | 1                                | 2, 3, 4, 5            |
| 2     | 1, 2                             | 3, 4, 5               |
| 3     | 1, 2, 3                          | 4, 5                  |
| 4     | 1, 2, 3, 4                       | 5                     |
| 5     | 1, 2, 3, 4, 5                    | none                  |

- d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

## K. VALIDATION RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figures 4 and 5. Peak height ratios for these calibrators appear in Tables 1A-B and 2A-B. Statistical parameters of plasma interday precision standard curve calibrators appear in Table 3A and of blood interday and intraday precision standard curve calibrators appear in Table 3B.

### 2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4A-B and 5A-B.

### 3. LLOQ

Results for this evaluation appear in Tables 6A-B.

### 4. RECOVERY

Results for this evaluation appear in Tables 7A-B.

### 6. STABILITY

a. System Stability: Results appear in Tables 8A-B.

b. Long Term Stability:\* Pooled plasma and blood samples spiked with halofantrine and WR 178,460 (as free bases) at four different concentrations were mixed on a rotator for one hour. The resulting samples were divided into 0.5 ml fractions, placed in culture tubes and stored in the freezer at -80°C until assayed for stability. Samples were assayed according to the method described in Study Report No. 4 dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma." Results appear in Table 9A-B.

c. Bench Top Stability: Results appear in Tables 10A-B.

d. Freeze/Thaw Stability: Results appear in Tables 11A-B.

### 8. BLIND SAMPLE ANALYSIS

Results appear in 12A-B for blood.

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\* Samples were assayed according to the method described in Study Report No. 4 dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma."

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
HALOFANTRINE (FREE BASE) PLASMA ASSAY,  
STUDY REPORT 17B

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |                          | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION |
|---------------------------|--------------------------|--------------------------|---------------------------|-----------------------------|
|                           | CONCENTRATION<br>(ng/ml) | CONCENTRATION<br>(ng/ml) |                           |                             |
| 0                         | 0                        | 0                        | 0                         | -                           |
| 1.04                      | 2.08                     | 0.072                    | 1.88 <sup>a</sup>         |                             |
| 2.08                      | 4.16                     | 0.169                    | 4.35 <sup>a</sup>         |                             |
| 4.16                      | 8.32                     | 0.321                    | 8.22 <sup>a</sup>         |                             |
| 8.32                      | 16.6                     | 0.654                    | 16.7 <sup>a</sup>         |                             |
| 16.64                     | 33.3                     | 1.303                    | 33.3 <sup>a</sup>         |                             |
| 33.28                     | 66.6                     | 2.830                    | 67.6 <sup>b</sup>         |                             |
| 66.56                     | 133                      | 5.525                    | 131 <sup>b</sup>          |                             |
| 133.12                    | 266                      | 11.279                   | 267 <sup>b</sup>          |                             |

Regression equations:\*\*\*

$y = 0.039228x - 0.001608$ ,  $r^2 = 0.9999$  (Low Range: 0 - 33.3 ng/ml)

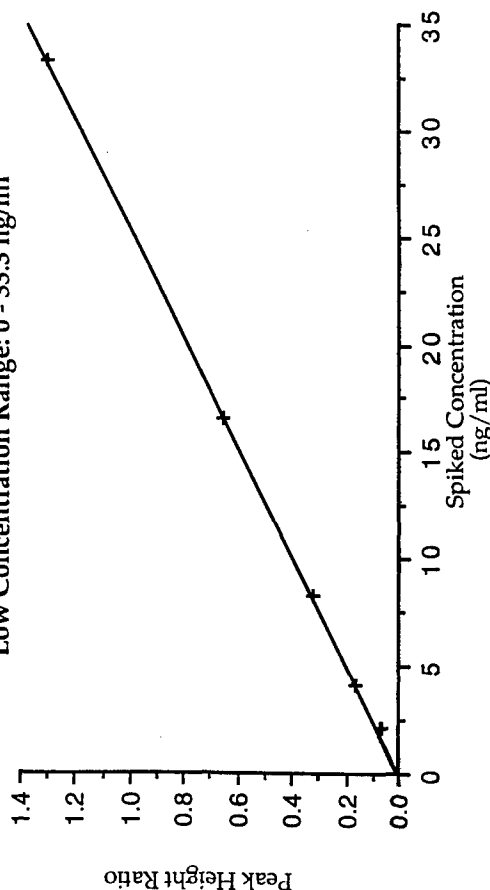
$y = 0.042393x - 0.035294$ ,  $r^2 = 0.9998$  (High Range: 0 - 266 ng/ml)

\* Into 0.5 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 33.3 ng/ml



High Concentration Range: 0 - 266 ng/ml

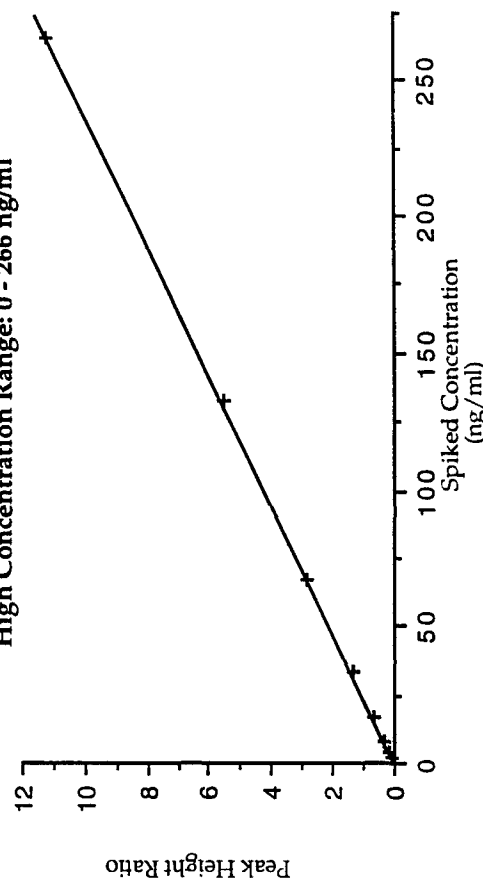


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
WR 178/460 (FREE BASE) PLASMA ASSAY,  
STUDY REPORT 17B

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |                          | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|--------------------------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) | CONCENTRATION<br>(ng/ml) |                           |                                        |
| 0                         | 0                        | 0                        | 0                         | -                                      |
| 1.04                      | 2.08                     | 0.125                    | 0.125                     | 1.97a                                  |
| 2.08                      | 4.16                     | 0.289                    | 0.289                     | 4.62a                                  |
| 4.16                      | 8.32                     | 0.523                    | 0.523                     | 8.41a                                  |
| 8.32                      | 16.6                     | 0.992                    | 0.992                     | 16.0a                                  |
| 16.64                     | 33.3                     | 2.075                    | 2.075                     | 33.5a                                  |
| 33.28                     | 66.6                     | 4.311                    | 4.311                     | 67.4b                                  |
| 66.56                     | 133                      | 8.375                    | 8.375                     | 131b                                   |
| 133.12                    | 266                      | 17.159                   | 17.159                    | 267b                                   |

Regression equations:\*\*\*

$a_y = 0.061782x + 0.003593$ ,  $r^2 = 0.9992$  (Low Range: 0 - 33.3 ng/ml)

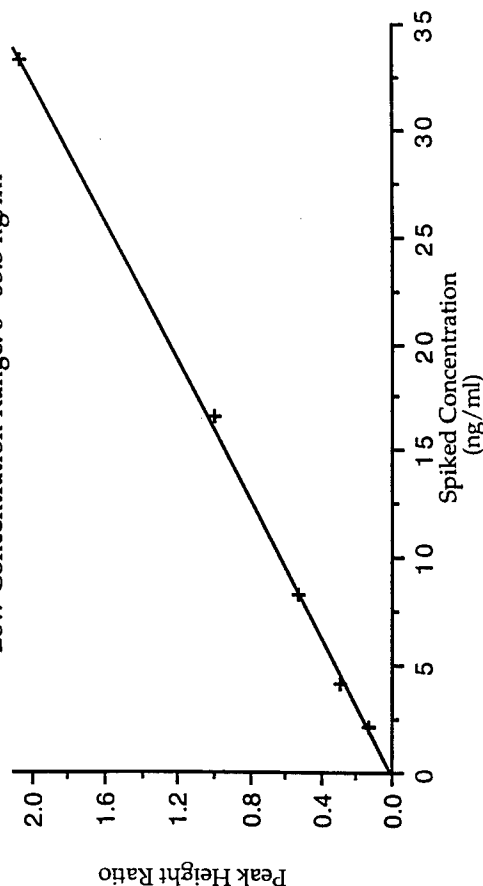
$b_y = 0.064353x - 0.029110$ ,  $r^2 = 0.9998$  (High Range: 0 - 266 ng/ml)

\* Into 0.5 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 33.3 ng/ml



High Concentration Range: 0 - 266 ng/ml

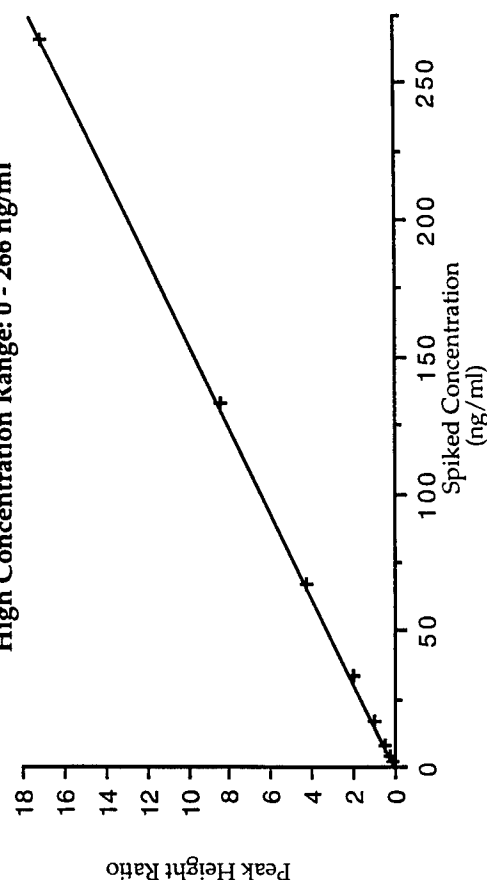


TABLE 2A: REPRESENTATIVE STANDARD CURVE FOR  
HALOFANTRINE (FREE BASE) BLOOD ASSAY,  
STUDY REPORT 17B

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |                          | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|--------------------------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) | CONCENTRATION<br>(ng/ml) |                           |                                        |
| 0                         | 0                        | 0                        | 0                         | -                                      |
| 0.511                     | 1.02                     | 0.006                    | 0.006                     | 0.978 <sup>a</sup>                     |
| 1.02                      | 2.04                     | 0.014                    | 0.014                     | 2.02 <sup>a</sup>                      |
| 1.53                      | 3.07                     | 0.022                    | 0.022                     | 3.07 <sup>a</sup>                      |
| 2.56                      | 5.11                     | 0.034                    | 0.034                     | 4.63 <sup>a</sup>                      |
| 5.11                      | 10.2                     | 0.080                    | 0.080                     | 10.6 <sup>a</sup>                      |
| 10.2                      | 20.4                     | 0.154                    | 0.154                     | 20.3 <sup>a</sup>                      |
| 20.4                      | 40.8                     | 0.297                    | 0.297                     | 41.0 <sup>b</sup>                      |
| 40.8                      | 81.6                     | 0.582                    | 0.582                     | 79.8 <sup>b</sup>                      |
| 61.2                      | 122                      | 0.849                    | 0.849                     | 116 <sup>b</sup>                       |
| 122                       | 245                      | 1.819                    | 1.819                     | 248 <sup>b</sup>                       |

Regression equations:

$a y = 0.007658x - 0.00149$ ,  $r^2 = 0.9985$  (Low Range: 0 - 20.4 ng/ml)

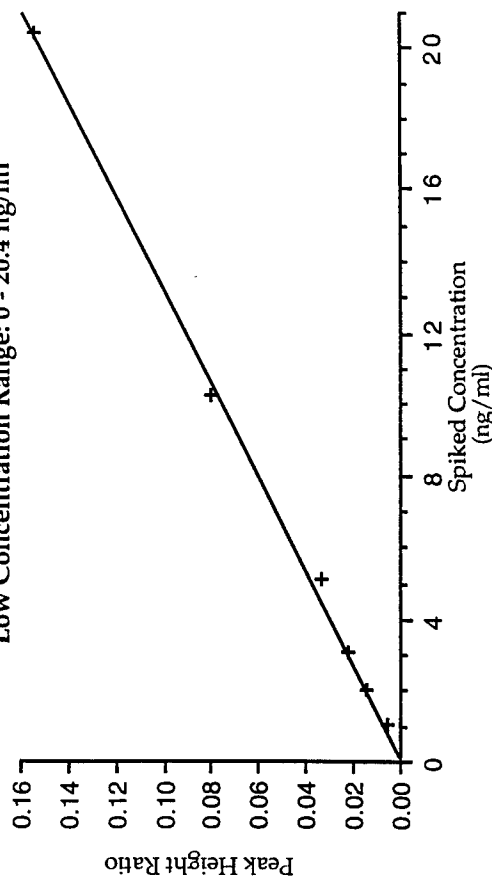
$b y = 0.007341x - 0.00391$ ,  $r^2 = 0.9991$  (High Range: 0 - 245 ng/ml)

\* Into 0.5 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 20.4 ng/ml



High Concentration Range: 0 - 245 ng/ml

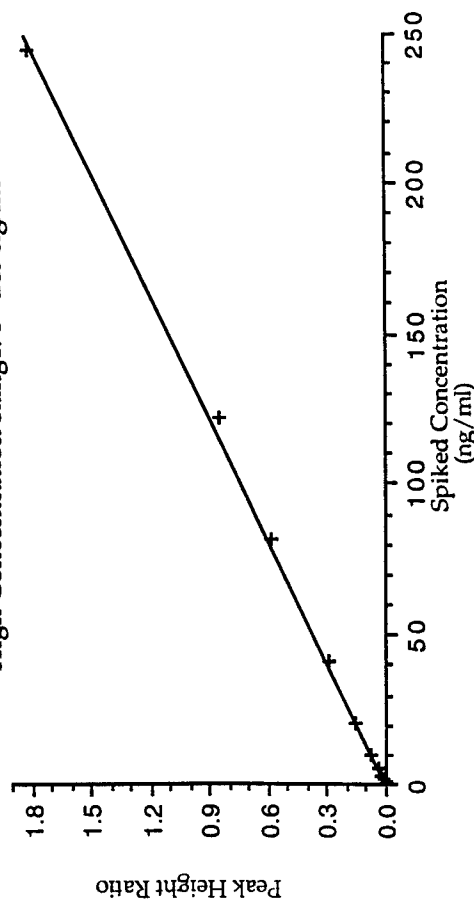


TABLE 2B: REPRESENTATIVE STANDARD CURVE FOR  
WR 178,460 (FREE BASE) BLOOD ASSAY,  
STUDY REPORT 17B

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD                 |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) | CURVE |                           |                                        |
| 0                         | 0                        | 0     | 0                         | -                                      |
| 0.482                     | 0.964                    | 0.964 | 0.010                     | 1.02 <sup>a</sup>                      |
| 0.964                     | 1.93                     | 1.93  | 0.017                     | 1.84 <sup>a</sup>                      |
| 1.45                      | 2.89                     | 2.89  | 0.026                     | 2.90 <sup>a</sup>                      |
| 2.41                      | 4.82                     | 4.82  | 0.041                     | 4.67 <sup>a</sup>                      |
| 4.82                      | 9.64                     | 9.64  | 0.088                     | 10.2 <sup>a</sup>                      |
| 9.64                      | 19.3                     | 19.3  | 0.163                     | 19.1 <sup>a</sup>                      |
| 19.3                      | 38.6                     | 38.6  | 0.331                     | 38.6 <sup>b</sup>                      |
| 38.6                      | 77.2                     | 77.2  | 0.653                     | 76.0 <sup>b</sup>                      |
| 57.9                      | 116                      | 116   | 0.974                     | 113 <sup>b</sup>                       |
| 116                       | 232                      | 232   | 2.013                     | 234 <sup>b</sup>                       |

Regression equations:\*\*\*

$a_y = 0.008483x + 0.001366$ ,  $r^2 = 0.9983$  (Low Range: 0 - 19.3 ng/ml)

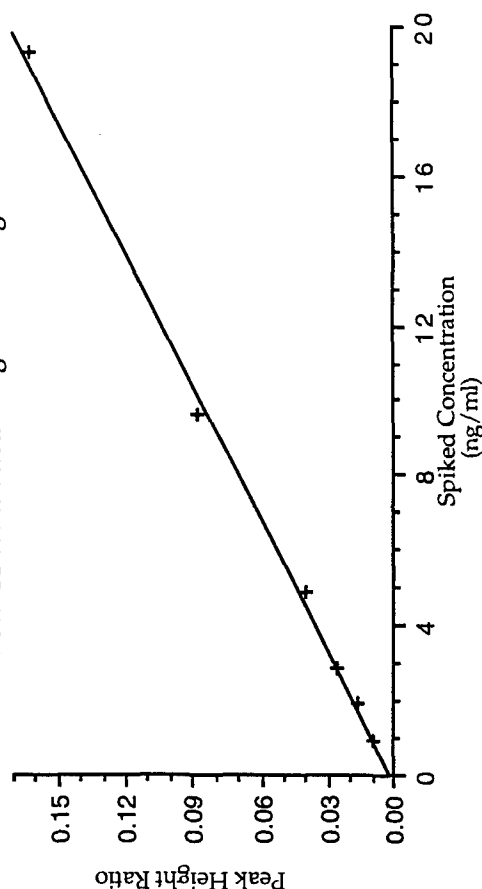
$b_y = 0.008621x - 0.00212$ ,  $r^2 = 0.9997$  (High Range: 0 - 232 ng/ml)

\* Into 0.5 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level (free base) concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 19.3 ng/ml



High Concentration Range: 0 - 232 ng/ml

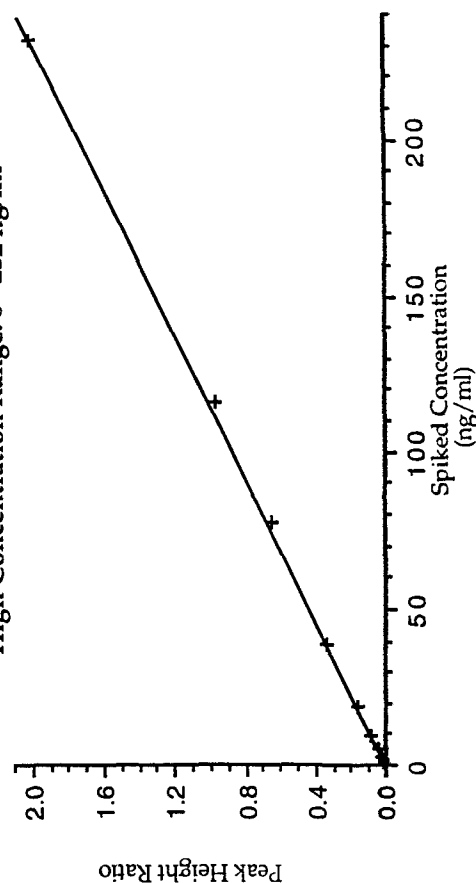


**TABLE 3A: INTERDAY PRECISION STANDARD CURVE CALIBRATOR  
STATISTICAL PARAMETERS FOR HALOFANTRINE PLASMA  
ASSAY, SR 17B**

| Spiked<br>Concentration<br>(ng/ml) | n | Mean<br>(ng/ml) | Standard<br>Deviation<br>(ng/ml) | Percent<br>C.V. | Percent<br>Deviation |
|------------------------------------|---|-----------------|----------------------------------|-----------------|----------------------|
| HALOFANTRINE                       |   |                 |                                  |                 |                      |
| 2.08                               | 6 | 1.81            | 0.217                            | 12.0            | -13.0                |
| 4.16                               | 6 | 4.06            | 0.306                            | 7.55            | -2.44                |
| 8.32                               | 6 | 8.39            | 0.791                            | 9.42            | 0.89                 |
| 16.6                               | 6 | 17.2            | 1.51                             | 8.76            | 3.71                 |
| 33.3                               | 6 | 33.0            | 0.574                            | 1.74            | -0.89                |
| 66.6                               | 6 | 63.7            | 3.00                             | 4.71            | -4.38                |
| 133                                | 6 | 131             | 7.36                             | 5.60            | -1.17                |
| 266                                | 6 | 268             | 4.12                             | 1.54            | 0.61                 |
| WR 178,460                         |   |                 |                                  |                 |                      |
| 2.08                               | 5 | 2.02            | 0.267                            | 13.3            | -3.06                |
| 4.16                               | 6 | 4.25            | 0.441                            | 10.4            | -3.06                |
| 8.32                               | 6 | 8.45            | 0.538                            | 6.37            | 2.06                 |
| 16.6                               | 6 | 16.7            | 0.889                            | 5.32            | 1.55                 |
| 33.3                               | 6 | 33.2            | 0.330                            | 0.99            | 0.62                 |
| 66.6                               | 6 | 65.5            | 2.02                             | 3.09            | -0.27                |
| 133                                | 6 | 132             | 7.69                             | 5.81            | -1.60                |
| 266                                | 6 | 267             | 4.00                             | 1.50            | -0.55                |

**TABLE 3B: PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS FOR HALOFANTRINE BLOOD ASSAY, SR 17B**

| Spiked<br>Concentration<br>(ng/ml) | n | Mean<br>(ng/ml) | Standard<br>Deviation<br>(ng/ml) | Percent<br>C.V. | Percent<br>Deviation |
|------------------------------------|---|-----------------|----------------------------------|-----------------|----------------------|
| HALOFANTRINE                       |   |                 |                                  |                 |                      |
| 1.02                               | 6 | 1.14            | 0.124                            | 10.9            | 11.9                 |
| 2.04                               | 7 | 2.08            | 0.117                            | 5.62            | 2.19                 |
| 3.07                               | 7 | 2.95            | 0.242                            | 8.20            | -3.79                |
| 5.11                               | 7 | 4.73            | 0.234                            | 4.96            | -7.42                |
| 10.2                               | 7 | 10.7            | 0.354                            | 3.33            | 4.45                 |
| 20.4                               | 7 | 20.3            | 0.157                            | 0.773           | -0.608               |
| 40.8                               | 7 | 40.2            | 2.86                             | 7.13            | -1.56                |
| 81.6                               | 7 | 79.7            | 3.53                             | 4.43            | -2.37                |
| 122                                | 7 | 121             | 6.42                             | 5.32            | -1.21                |
| 245                                | 7 | 246             | 3.29                             | 1.34            | 0.604                |
| WR 178,460                         |   |                 |                                  |                 |                      |
| 0.964                              | 7 | 0.994           | 0.068                            | 6.79            | 3.13                 |
| 1.93                               | 7 | 1.96            | 0.193                            | 9.83            | 1.54                 |
| 2.89                               | 7 | 2.94            | 0.177                            | 6.03            | 1.75                 |
| 4.89                               | 7 | 4.88            | 0.222                            | 4.54            | 1.24                 |
| 9.64                               | 7 | 9.65            | 0.478                            | 4.95            | 0.092                |
| 19.3                               | 7 | 19.3            | 0.197                            | 1.02            | -0.163               |
| 38.6                               | 7 | 38.5            | 1.56                             | 4.04            | -0.245               |
| 77.2                               | 7 | 75.3            | 1.58                             | 2.09            | -2.44                |
| 116                                | 7 | 116             | 2.83                             | 2.45            | -0.107               |
| 232                                | 7 | 233             | 1.76                             | 0.756           | 0.321                |

TABLE 4A: PRECISION OF HALOFANTRINE HUMAN PLASMA ASSAY

Inter-Run Precision Halofantrine Free Base

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |        |      |
|---------------------------------|------------------|-------------------------------|-------|--------|------|
|                                 |                  | 4.04                          | 10.1  | 40.4   | 129  |
| Measured Concentrations (ng/mL) |                  |                               |       |        |      |
| 1                               | 1                | 4.84                          | 10.0  | 43.1   | 155  |
|                                 | 2                | 4.58                          | 9.65  | 40.7   | 126  |
| 2                               | 1                | 4.27                          | 9.26  | 39.5   | 122  |
|                                 | 2                | 4.66                          | 11.2  | 46.2   | 116  |
| 3                               | 1                | 3.64                          | 10.7  | 40.1   | 143  |
|                                 | 2                | 3.24                          | 9.67  | 41.6   | 142  |
| 4                               | 1                | 3.53                          | 10.1  | 42.5   | 133  |
|                                 | 2                | 3.33                          | 10.7  | 38.6   | 137  |
| 5                               | 1                | 3.31                          | 9.61  | 33.3   | 124  |
|                                 | 2                | 3.99                          | 8.68  | 38.1   | 115  |
| 6                               | 1                | 3.88                          | bc    | 38.8   | 135  |
|                                 | 2                | 4.29                          | 10.1  | 38.2   | 150  |
| n                               |                  | 12                            | 11    | 12     | 12   |
| Mean                            |                  | 3.96                          | 9.97  | 40.1   | 133  |
| S.D.                            |                  | 0.563                         | 0.715 | 3.2    | 12.9 |
| Percent C.V.                    |                  | 14.2                          | 7.17  | 8.00   | 9.69 |
| Percent R.E.                    |                  | -1.87                         | -1.25 | -0.825 | 3.31 |

Intra-Run Precision Pyridostigmine Cation

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 2.04                          | 10.2  | 61.2  | 102   |
| Measured Concentrations (ng/mL) |                  |                               |       |       |       |
| 7                               | 1                | 1.56                          | 9.80  | 51.4  | 80.5  |
|                                 | 2                | 1.73                          | 9.94  | 52.1  | 87.0  |
|                                 | 3                | 1.94                          | 9.77  | 52.9  | 93.7  |
|                                 | 4                | 1.94                          | 10.4  | 48.1  | 83.6  |
|                                 | 5                | 1.89                          | 11.1  | 53.1  | 89.4  |
|                                 | 6                | 1.98                          | 9.72  | 49.7  | 90.9  |
| n                               |                  | 6                             | 6     | 6     | 6     |
| Mean                            |                  | 1.84                          | 10.1  | 51.2  | 87.5  |
| S.D.                            |                  | 0.161                         | 0.54  | 1.96  | 4.86  |
| Percent C.V.                    |                  | 8.75                          | 5.35  | 3.83  | 5.55  |
| Percent R.E.                    |                  | -9.80                         | -1.00 | -16.3 | -14.2 |

bc = chromatogram unacceptable.

TABLE 4B: PRECISION OF HALOFANTRINE HUMAN PLASMA ASSAY

Inter-Run Precision WR 178,460 Free Base

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |        |      |
|---------------------------------|------------------|-------------------------------|-------|--------|------|
|                                 |                  | 4.16                          | 10.4  | 41.6   | 133  |
| Measured Concentrations (ng/mL) |                  |                               |       |        |      |
| 1                               | 1                | 4.48                          | 10.8  | 43.3   | 153  |
|                                 | 2                | 4.60                          | 10.4  | 41.9   | 131  |
| 2                               | 1                | 4.18                          | 10.6  | 42.3   | 126  |
|                                 | 2                | bc                            | 11.4  | 47.1   | 126  |
| 3                               | 1                | 3.18                          | 10.3  | 42.0   | 146  |
|                                 | 2                | 4.27                          | 10.5  | 41.7   | 143  |
| 4                               | 1                | bc                            | 10.8  | 44.0   | 136  |
|                                 | 2                | 4.49                          | 10.2  | 41.0   | 138  |
| 5                               | 1                | 4.00                          | bc    | 34.7   | 124  |
|                                 | 2                | 4.16                          | 9.69  | 38.1   | 116  |
| 6                               | 1                | 4.62                          | bc    | 42.1   | 138  |
|                                 | 2                | 5.26                          | 10.3  | 40.2   | 150  |
| n                               |                  | 10                            | 10    | 12     | 12   |
| Mean                            |                  | 4.32                          | 10.5  | 41.5   | 136  |
| S.D.                            |                  | 0.532                         | 0.441 | 3.04   | 11.1 |
| Percent C.V.                    |                  | 12.3                          | 4.19  | 7.32   | 8.20 |
| Percent R.E.                    |                  | 3.91                          | 1.04  | -0.149 | 1.94 |

Intra-Run Precision WR 178,460 Free Base

| Validation                      | QC         | Spiked Concentrations (ng/mL) |       |       |       |
|---------------------------------|------------|-------------------------------|-------|-------|-------|
| Run No.                         | Sample No. | 1.93                          | 9.64  | 57.9  | 96.5  |
| Measured Concentrations (ng/mL) |            |                               |       |       |       |
| 7                               | 1          | 2.13                          | 10.1  | 56.8  | 92.9  |
|                                 | 2          | 1.98                          | 10.2  | 56.1  | 93.1  |
|                                 | 3          | 1.98                          | 9.95  | 56.9  | 98.1  |
|                                 | 4          | 1.93                          | 9.89  | 54.6  | 94.0  |
|                                 | 5          | 1.98                          | 10.1  | 55.4  | 96.6  |
|                                 | 6          | 2.30                          | 9.71  | 55.5  | 98.1  |
| n                               |            | 6                             | 6     | 6     | 6     |
| Mean                            |            | 2.05                          | 10    | 55.9  | 95.5  |
| S.D.                            |            | 0.142                         | 0.194 | 0.906 | 2.45  |
| Percent C.V.                    |            | 6.95                          | 1.94  | 1.62  | 2.56  |
| Percent R.E.                    |            | 6.11                          | 3.78  | -3.49 | -1.08 |

bc = chromatogram unacceptable.

TABLE 5A: PRECISION OF HALOFANTRINE HUMAN BLOOD ASSAY

Inter-Run Precision Halofantrine Free Base

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |      |       |       |
|---------------------------------|------------------|-------------------------------|------|-------|-------|
|                                 |                  | 4.16                          | 10.4 | 41.6  | 133   |
| Measured Concentrations (ng/mL) |                  |                               |      |       |       |
| 1                               | 1                | 1.71                          | 10.8 | 60.4  | 103   |
|                                 | 2                | 2.01                          | 8.35 | 59.2  | 101   |
| 2                               | 1                | 2.43                          | 11.7 | 60.7  | 105   |
|                                 | 2                | 2.11                          | 10.4 | 66.0  | 90.8  |
| 3                               | 1                | 2.15                          | 11.0 | 62.9  | 106   |
|                                 | 2                | 2.02                          | 11.2 | 62.0  | 99.2  |
| 4                               | 1                | 2.19                          | 11.0 | 60.1  | 102   |
|                                 | 2                | 1.91                          | 9.82 | 59.7  | 104   |
| 5                               | 1                | 1.66                          | 10.7 | 52.5  | 87.5  |
|                                 | 2                | 1.96                          | 12.6 | 59.9  | 93.4  |
| 6                               | 1                | 1.91                          | 9.85 | 62.8  | 89.9  |
|                                 | 2                | bc                            | 11.2 | 58.1  | 104   |
| n                               |                  | 11                            | 12   | 12    | 12    |
| Mean                            |                  | 2.01                          | 10.7 | 60.4  | 98.8  |
| S.D.                            |                  | 0.218                         | 1.06 | 3.25  | 6.59  |
| Percent C.V.                    |                  | 10.8                          | 9.91 | 5.38  | 6.67  |
| Percent R.E.                    |                  | -1.69                         | 5.08 | -1.38 | -3.12 |

Intra-Run Precision Halofantrine Free Base

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |       |      |
|---------------------------------|------------------|-------------------------------|-------|-------|------|
|                                 |                  | 2.04                          | 10.2  | 61.2  | 102  |
| Measured Concentrations (ng/mL) |                  |                               |       |       |      |
| 7                               | 1                | 1.86                          | 10.4  | 53.4  | 90.8 |
|                                 | 2                | 2.00                          | 7.68  | 58.4  | 107  |
|                                 | 3                | 2.00                          | 9.75  | 53.3  | 89.5 |
|                                 | 4                | 1.58                          | bc    | 64.0  | 103  |
|                                 | 5                | 2.14                          | 7.68  | 57.4  | 109  |
|                                 | 6                | 1.72                          | 11.0  | 46.2  | 113  |
| n                               |                  | 6                             | 5     | 6     | 6    |
| Mean                            |                  | 1.88                          | 9.30  | 55.5  | 102  |
| S.D.                            |                  | 0.206                         | 1.55  | 6.00  | 9.77 |
| Percent C.V.                    |                  | 11.0                          | 16.7  | 10.8  | 9.58 |
| Percent R.E.                    |                  | -7.84                         | -8.82 | -9.31 | 0    |

bc = chromatogram unacceptable.

TABLE 5B: PRECISION OF HALOFANTRINE HUMAN BLOOD ASSAY

Inter-Run Precision WR 178,460 Free Base

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |         |        |       |
|---------------------------------|------------------|-------------------------------|---------|--------|-------|
|                                 |                  | 1.93                          | 9.64    | 57.9   | 96.5  |
| Measured Concentrations (ng/mL) |                  |                               |         |        |       |
| 1                               | 1                | 1.60                          | 9.26    | 60.4   | 99.5  |
|                                 | 2                | 1.85                          | 9.26    | 58.5   | 95.9  |
| 2                               | 1                | 1.69                          | 9.38    | 58.7   | 98.8  |
|                                 | 2                | 1.81                          | 9.63    | 57.9   | 94.0  |
| 3                               | 1                | 1.84                          | 10.2    | 58.4   | 97.8  |
|                                 | 2                | 1.73                          | 9.98    | 57.2   | 96.4  |
| 4                               | 1                | 2.02                          | 9.80    | 57.1   | 96.0  |
|                                 | 2                | 2.02                          | 9.31    | 58.4   | 99.2  |
| 5                               | 1                | 1.85                          | 9.35    | 53.3   | 88.6  |
|                                 | 2                | 1.97                          | 9.84    | 56.9   | 92.9  |
| 6                               | 1                | 2.00                          | 9.55    | 58.4   | 93.6  |
|                                 | 2                | bc                            | 10.0    | 57.2   | 93.5  |
| n                               |                  | 11                            | 12      | 12     | 12    |
| Mean                            |                  | 1.93                          | 9.63    | 57.7   | 95.5  |
| S.D.                            |                  | 0.142                         | 0.331   | 1.67   | 3.17  |
| Percent C.V.                    |                  | 7.67                          | 3.44    | 2.90   | 3.32  |
| Percent R.E.                    |                  | -4.04                         | -0.0765 | -0.351 | -1.02 |

Intra-Run Precision WR 178,460 Free Base

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 2.04                          | 10.2  | 61.2  | 102   |
| Measured Concentrations (ng/mL) |                  |                               |       |       |       |
| 7                               | 1                | 1.70                          | 10.0  | 54.3  | 92.2  |
|                                 | 2                | 1.83                          | 9.20  | 55.0  | 97.6  |
|                                 | 3                | 1.83                          | 9.83  | 51.5  | 93.1  |
|                                 | 4                | 1.83                          | bc    | 58.1  | 97.9  |
|                                 | 5                | 1.83                          | 9.33  | 56.8  | 99.3  |
|                                 | 6                | 1.95                          | 10.1  | 55.9  | 99.5  |
| n                               |                  |                               |       |       |       |
| Mean                            |                  | 1.83                          | 9.68  | 55.3  | 96.6  |
| S.D.                            |                  | 0.079                         | 0.389 | 2.29  | 3.15  |
| Percent C.V.                    |                  | 4.33                          | 4.02  | 4.14  | 3.26  |
| Percent R.E.                    |                  | -5.34                         | 0.400 | -4.53 | 0.100 |

bc = chromatogram unacceptable.

**TABLE 6A: LOW POINT VALIDATION OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) HUMAN PLASMA ASSAY**

|                      | HALOFANTRINE<br>(free base)<br>(2.08 ng/ml) |          | WR 178,460<br>(free base)<br>(2.08 ng/ml) |          |
|----------------------|---------------------------------------------|----------|-------------------------------------------|----------|
| Spiked Concentration | Measured Concentrations<br>(ng/ml)          |          |                                           |          |
|                      | Interday                                    | Intraday | Interday                                  | Intraday |
|                      | 1.98                                        | 2.06     | 1.86                                      | 2.13     |
|                      | 1.53                                        | 1.51     | b.c.                                      | 2.16     |
|                      | 1.79                                        | 1.80     | 2.06                                      | 2.22     |
|                      | 1.88                                        | 2.48     | 1.97                                      | 2.04     |
|                      | 1.59                                        | 1.99     | 1.75                                      | 2.01     |
|                      | 2.08                                        | bc       | 2.45                                      | b.c.     |
|                      | 1.81                                        | 1.97     | 2.02                                      | 2.11     |
| Mean                 |                                             |          |                                           |          |
| Standard Deviation   | 0.217                                       | 0.36     | 0.267                                     | 0.09     |
| Percent CV           | 12.0                                        | 18.2     | 13.3                                      | 4.07     |
| Percent Error        | -13.0                                       | -5.67    | -3.06                                     | 1.52     |

**TABLE 6B: LOW POINT VALIDATION OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) HUMAN BLOOD ASSAY**

|                      | HALOFANTRINE                       |          | WR 178,460    |          |
|----------------------|------------------------------------|----------|---------------|----------|
|                      | (free base)                        |          | (free base)   |          |
| Spiked Concentration | (1.02 ng/ml)                       |          | (0.964 ng/ml) |          |
|                      | Measured Concentrations<br>(ng/ml) |          |               |          |
|                      | Interday                           | Intraday | Interday      | Intraday |
|                      | 1.10                               | 1.28     | 1.09          | b.c.     |
|                      | b.c.                               | 1.01     | 0.928         | 0.798    |
|                      | 0.978                              | 1.01     | 1.02          | 0.923    |
|                      | 1.19                               | b.c.     | 0.927         | 0.923    |
|                      | 1.07                               | 1.14     | 0.971         | 1.05     |
|                      | 1.34                               | 1.14     | 0.951         | 0.923    |
| Mean                 | 1.14                               | 1.12     | 0.980         | 0.923    |
| Standard Deviation   | 0.138                              | 0.112    | 0.062         | 0.088    |
| Percent CV           | 12.2                               | 10.1     | 6.35          | 9.57     |
| Percent Error        | 11.4                               | 9.45     | 1.70          | -4.28    |

bc = unacceptable chromatogram.

**TABLE 7A: RECOVERIES OF HALOFANTRINE AND WR 178,460 FROM HUMAN PLASMA**

| SAMPLE<br>ID | SPIKED<br>CONCENTRATION<br>Range | (ng/ml) | PEAK HEIGHT RATIO |              | MEAN<br>PERCENT<br>RECOVERY |
|--------------|----------------------------------|---------|-------------------|--------------|-----------------------------|
|              |                                  |         | SOLVENT           | PLASMA       |                             |
| HALOFANTRINE |                                  |         |                   |              |                             |
| 1            | X Low                            | 2.04    | 0.011             | 0.005        | 55.2                        |
| 2            |                                  |         | 0.007             | 0.005        |                             |
| 3            |                                  |         | 0.011             | 0.006        |                             |
| Mean (± SD)  |                                  |         | 0.010 ±0.002      | 0.005 ±0.001 |                             |
| 1            | Low                              | 10.2    | 0.074             | 0.035        | 52.4                        |
| 2            |                                  |         | 0.064             | 0.023        |                             |
| 3            |                                  |         | 0.068             | 0.050        |                             |
| Mean (± SD)  |                                  |         | 0.069 ±0.005      | 0.036 ±0.014 |                             |
| 1            | Medium                           | 61.2    | 0.416             | 0.193        | 35.7                        |
| 2            |                                  |         | 0.421             | 0.060        |                             |
| 3            |                                  |         | 0.415             | 0.194        |                             |
| Mean (± SD)  |                                  |         | 0.417 ±0.003      | 0.149 ±0.077 |                             |
| 1            | High                             | 102     | 0.685             | 0.278        | 42.5                        |
| 2            |                                  |         | 0.701             | 0.342        |                             |
| 3            |                                  |         | 0.694             | 0.263        |                             |
| Mean (± SD)  |                                  |         | 0.693 ±0.008      | 0.294 ±0.042 |                             |
| AVERAGE =    |                                  |         |                   |              | 46.4                        |
| WR 178,460   |                                  |         |                   |              |                             |
| 1            | X-Low                            | 1.93    | 0.011             | 0.011        | 105.9                       |
| 2            |                                  |         | 0.010             | 0.010        |                             |
| 3            |                                  |         | 0.013             | 0.015        |                             |
| Mean (± SD)  |                                  |         | 0.011 ±0.002      | 0.012 ±0.003 |                             |
| 1            | Low                              | 9.64    | 0.075             | 0.060        | 78.4                        |
| 2            |                                  |         | 0.077             | 0.056        |                             |
| 3            |                                  |         | 0.079             | 0.065        |                             |
| Mean (± SD)  |                                  |         | 0.077 ±0.002      | 0.060 ±0.005 |                             |
| 1            | Medium                           | 57.9    | 0.454             | 0.335        | 72.2                        |
| 2            |                                  |         | 0.458             | 0.316        |                             |
| 3            |                                  |         | 0.469             | 0.346        |                             |
| Mean (± SD)  |                                  |         | 0.460 ±0.008      | 0.332 ±0.015 |                             |
| 1            | High                             | 96.5    | 0.748             | 0.546        | 75.1                        |
| 2            |                                  |         | 0.774             | 0.636        |                             |
| 3            |                                  |         | 0.748             | 0.522        |                             |
| Mean (± SD)  |                                  |         | 0.757 ±0.015      | 0.568 ±0.060 |                             |
| AVERAGE =    |                                  |         |                   |              | 82.9                        |

**TABLE 7B: RECOVERIES OF HALOFANTRINE AND WR 178,460 FROM HUMAN BLOOD**

| SAMPLE ID           | SPIKED CONCENTRATION<br>Range (ng/ml) |      | PEAK HEIGHT RATIO |              | MEAN PERCENT RECOVERY |
|---------------------|---------------------------------------|------|-------------------|--------------|-----------------------|
|                     |                                       |      | SOLVENT           | PLASMA       |                       |
| <u>HALOFANTRINE</u> |                                       |      |                   |              |                       |
| 1                   | X-Low                                 | 2.04 | 0.062             | 0.043        | 80.0                  |
| 2                   |                                       |      | bc                | 0.054        |                       |
| 3                   |                                       |      | 0.063             | 0.053        |                       |
| Mean (± SD)         |                                       |      | 0.063 ±0.001      | 0.050 ±0.006 |                       |
| 1                   | Low                                   | 10.2 | 0.382             | 0.345        | 79.2                  |
| 2                   |                                       |      | 0.399             | 0.269        |                       |
| 3                   |                                       |      | 0.389             | 0.313        |                       |
| Mean (± SD)         |                                       |      | 0.390 ±0.009      | 0.309 ±0.038 |                       |
| 1                   | Medium                                | 61.2 | 2.263             | 1.842        | 82.0                  |
| 2                   |                                       |      | 2.357             | 2.027        |                       |
| 3                   |                                       |      | 2.466             | 1.944        |                       |
| Mean (± SD)         |                                       |      | 2.362 ±0.102      | 1.938 ±0.093 |                       |
| 1                   | High                                  | 102  | 3.954             | 3.227        | 81.9                  |
| 2                   |                                       |      | 3.766             | 3.340        |                       |
| 3                   |                                       |      | 3.989             | 3.020        |                       |
| Mean (± SD)         |                                       |      | 3.903 ±0.120      | 3.196 ±0.162 |                       |
| AVERAGE =           |                                       |      |                   |              | 80.8                  |
| <u>WR 178,460</u>   |                                       |      |                   |              |                       |
| 1                   | X-Low                                 | 1.93 | 0.087             | 0.078        | 88.5                  |
| 2                   |                                       |      | bc                | 0.074        |                       |
| 3                   |                                       |      | 0.084             | 0.075        |                       |
| Mean (± SD)         |                                       |      | 0.086 ±0.002      | 0.076 ±0.002 |                       |
| 1                   | Low                                   | 9.64 | 0.436             | 0.379        | 85.8                  |
| 2                   |                                       |      | 0.416             | 0.357        |                       |
| 3                   |                                       |      | 0.416             | 0.352        |                       |
| Mean (± SD)         |                                       |      | 0.423 ±0.012      | 0.363 ±0.014 |                       |
| 1                   | Medium                                | 57.9 | 2.512             | 2.193        | 85.8                  |
| 2                   |                                       |      | 2.607             | 2.257        |                       |
| 3                   |                                       |      | 2.708             | 2.266        |                       |
| Mean (± SD)         |                                       |      | 2.609 ±0.098      | 2.239 ±0.040 |                       |
| 1                   | High                                  | 96.5 | 4.457             | 3.971        | 90.4                  |
| 2                   |                                       |      | 4.191             | 3.980        |                       |
| 3                   |                                       |      | 4.287             | 3.737        |                       |
| Mean (± SD)         |                                       |      | 4.312 ±0.135      | 3.896 ±0.138 |                       |
| AVERAGE =           |                                       |      |                   |              | 87.6                  |

bc = unacceptable chromatogram.

**TABLE 8A: SYSTEM STABILITY IN PREPARED HUMAN PLASMA****Concentration for Prepared Biological Samples Stored at Room Temperature**

Halofantrine (Free Base)

|                       |  | CONCENTRATION#<br>(ng/ml) |      |      |      |
|-----------------------|--|---------------------------|------|------|------|
| Spiked Concentration: |  | 2.04                      | 10.2 | 61.2 | 102  |
| TIME STORED           |  |                           |      |      |      |
| 0 day                 |  | 2.08                      | 10.8 | 60.0 | 98.4 |
| 1 day                 |  | 2.21                      | 11.4 | 63.1 | 106  |

WR 178,460 (Free Base)

|                       |  |      |      |      |      |
|-----------------------|--|------|------|------|------|
| Spiked Concentration: |  | 1.93 | 9.64 | 57.9 | 96.5 |
| TIME STORED           |  |      |      |      |      |
| 0 day                 |  | 2.09 | 9.81 | 56.7 | 94.8 |
| 1 day                 |  | 2.26 | 10.2 | 60.3 | 101  |

**TABLE 8B: SYSTEM STABILITY IN PREPARED HUMAN BLOOD****Concentration for Prepared Biological Samples Stored at Room Temperature**

Halofantrine (Free Base)

|                       |  | CONCENTRATION#<br>(ng/ml) |      |      |      |
|-----------------------|--|---------------------------|------|------|------|
| Spiked Concentration: |  | 2.04                      | 10.2 | 61.2 | 102  |
| TIME STORED           |  |                           |      |      |      |
| 0 day                 |  | 2.07                      | 9.72 | 59.6 | 102  |
| 1 day                 |  | 2.07                      | 9.85 | 60.1 | 96.7 |

WR 178,460 (Free Base)

|                       |  |      |      |      |      |
|-----------------------|--|------|------|------|------|
| Spiked Concentration: |  | 1.93 | 9.64 | 57.9 | 96.5 |
| TIME STORED           |  |      |      |      |      |
| 0 day                 |  | 1.86 | 9.63 | 56.2 | 95.2 |
| 1 day                 |  | 2.08 | 9.33 | 56.9 | 93.7 |

#Measured concentrations are averages of two analyses.

**TABLE 9A: STABILITY OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN PLASMA<sup>#</sup>**

**HALOFANTRINE (FREE BASE) CONCENTRATION IN PLASMA STORED AT -80°C**

|                       |  | CONCENTRATION<br>(ng/ml) |      |      |      |
|-----------------------|--|--------------------------|------|------|------|
| Spiked Concentration: |  | 4.50                     | 10.8 | 28.8 | 63.0 |
| DAYS STORED           |  |                          |      |      |      |
| 0                     |  | 4.68                     | 9.96 | 26.9 | 59.0 |
| 1                     |  | 4.76                     | 11.2 | 26.2 | 56.9 |
| 2                     |  | 4.20                     | 9.76 | 25.8 | 57.6 |
| 29                    |  | 4.85                     | 10.8 | 24.5 | 56.2 |
| 60                    |  | 3.99                     | 10.3 | 30.1 | 63.5 |
| 92                    |  | 3.68                     | 9.41 | 21.4 | 45.6 |
| 112                   |  | 3.81                     | 8.91 | 23.4 | 55.2 |
| 126                   |  | 4.02                     | 10.2 | 27.3 | 55.4 |
| MEAN                  |  | 4.25                     | 10.1 | 25.7 | 56.2 |

**WR 178,460 (FREE BASE) CONCENTRATION IN PLASMA STORED AT -80°C**

|                       |  | CONCENTRATION<br>(ng/ml) |      |      |      |
|-----------------------|--|--------------------------|------|------|------|
| Spiked Concentration: |  | 7.00                     | 16.8 | 44.8 | 98.0 |
| DAYS STORED           |  |                          |      |      |      |
| 0                     |  | 7.78                     | 17.4 | 50.7 | 101  |
| 1                     |  | 6.30                     | 17.3 | 47.8 | 104  |
| 2                     |  | 7.73                     | 21.6 | 51.2 | 108  |
| 29                    |  | 8.42                     | 19.7 | 46.7 | 104  |
| 60                    |  | 6.91                     | 16.6 | 48.4 | 100  |
| 92                    |  | 5.34                     | 15.3 | 45.0 | 95.4 |
| 112                   |  | 7.16                     | 16.6 | 44.6 | 106  |
| 126                   |  | 7.40                     | 18.1 | 45.7 | 86.4 |
| MEAN                  |  | 7.13                     | 17.8 | 47.5 | 101  |

Concentrations are means of multiple (usu. 3) analyses.

<sup>#</sup> Table taken from Project Status Report No. 7, dated Dec. 23, 1987 from data obtained according to the method described in Study Report No. 4, dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma."

**TABLE 9B: STABILITY OF HALOFANTRINE AND WR 178,460 (AS FREE BASES) IN BLOOD<sup>#</sup>**

| HALOFANTRINE (FREE BASE) CONCENTRATION IN BLOOD STORED AT -80°C |      |                          |      |      |
|-----------------------------------------------------------------|------|--------------------------|------|------|
|                                                                 |      | CONCENTRATION<br>(ng/ml) |      |      |
| Spiked Concentration:                                           | 4.50 | 10.8                     | 28.8 | 63.0 |
| DAYS STORED                                                     |      |                          |      |      |
| 0                                                               | 5.57 | 12.0                     | 31.2 | 65.0 |
| 1                                                               | 5.29 | 11.2                     | 28.9 | 61.4 |
| 4                                                               | 4.62 | 9.98                     | 28.5 | 63.4 |
| 29                                                              | 3.72 | 9.25                     | 24.4 | 51.7 |
| 61                                                              | 4.47 | 10.7                     | 27.9 | 60.3 |
| 90                                                              | 4.86 | 11.5                     | 28.2 | 45.9 |
| 110                                                             | 5.30 | 13.9                     | 30.5 | 55.5 |
| 128                                                             | 3.99 | 11.6                     | 27.3 | 60.1 |
| MEAN                                                            | 4.73 | 11.3                     | 28.4 | 57.9 |

| WR 178,460 (FREE BASE) CONCENTRATION IN BLOOD STORED AT -80°C |      |                          |      |      |
|---------------------------------------------------------------|------|--------------------------|------|------|
|                                                               |      | CONCENTRATION<br>(ng/ml) |      |      |
| Spiked Concentration:                                         | 7.00 | 16.8                     | 44.8 | 98.0 |
| DAYS STORED                                                   |      |                          |      |      |
| 0                                                             | 8.62 | 19.1                     | 50.6 | 102  |
| 1                                                             | 10.1 | 17.6                     | 39.7 | 107  |
| 4                                                             | 6.73 | 17.7                     | 47.4 | 101  |
| 29                                                            | 7.84 | 21.2                     | 49.5 | 108  |
| 61                                                            | 6.66 | 17.1                     | 44.2 | 94.2 |
| 90                                                            | 8.84 | 20.0                     | 46.1 | 69.2 |
| 110                                                           | 7.77 | 16.5                     | 44.9 | 85.6 |
| 128                                                           | 5.80 | 18.2                     | 45.1 | 90.5 |
| MEAN                                                          | 7.80 | 18.4                     | 45.9 | 94.7 |

Concentrations are means of multiple (usu. 3) analyses.

<sup>#</sup> Table taken from Project Status Report No. 7, dated Dec. 23, 1987 from data obtained according to the method described in Study Report No. 4, dated Aug. 23, 1985 and titled "Ion-Paired Liquid Chromatographic Method for the Analysis of Halofantrine (WR 171,669) and its Putative Metabolite (WR 178,460) in Blood and Plasma."

**TABLE 10A: BENCH TOP STABILITY OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) IN SPIKED HUMAN PLASMA #**

| Spiked<br>Concentration | HALOFANTRINE<br>(free base) |                       | WR 178,460<br>(free base) |                       |
|-------------------------|-----------------------------|-----------------------|---------------------------|-----------------------|
|                         | Low<br>Concentration        | High<br>Concentration | Low<br>Concentration      | High<br>Concentration |
|                         | (10.2 ng/ml)                | (102 ng/ml)           | (9.64 ng/ml)              | (96.5 ng/ml)          |
| HOURS                   |                             |                       |                           |                       |
| 0                       | 10.4                        | 103                   | 9.97                      | 93.9                  |
| 1                       | 11.1                        | 103                   | 9.63                      | 94.3                  |
| 2                       | 10.4                        | 103                   | 9.53                      | 96.9                  |
| 4                       | 10.5                        | 107                   | 9.63                      | 96.2                  |

**TABLE 10B: BENCH TOP STABILITY OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) IN SPIKED HUMAN BLOOD ‡**

| Spiked<br>Concentration | HALOFANTRINE<br>(free base) |                       | WR 178,460<br>(free base) |                       |
|-------------------------|-----------------------------|-----------------------|---------------------------|-----------------------|
|                         | Low<br>Concentration        | High<br>Concentration | Low<br>Concentration      | High<br>Concentration |
|                         | (10.2 ng/ml)                | (102 ng/ml)           | (9.64 ng/ml)              | (96.5 ng/ml)          |
| HOURS                   |                             |                       |                           |                       |
| 0                       | 10.4                        | 101                   | 9.73                      | 96.1                  |
| 1                       | 9.57                        | 92.4                  | 8.99                      | 93.4                  |
| 2                       | 10.3                        | 103                   | 10.1                      | 94.5                  |
| 4                       | 10.3                        | 102                   | 9.34                      | 95.5                  |

# Measured concentrations are averages of two analyses.

‡ Measured concentrations are averages of three analyses.

**TABLE 11A: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON HALOFANTRINE AND WR 178,460 (AS FREE BASES) SPIKED HUMAN PLASMA SAMPLES<sup>#</sup>**

| Spiked<br>Concentration | HALOFANTRINE<br>(free base) |                       | WR 178,460<br>(free base) |                       |
|-------------------------|-----------------------------|-----------------------|---------------------------|-----------------------|
|                         | Low<br>Concentration        | High<br>Concentration | Low<br>Concentration      | High<br>Concentration |
|                         | (10.2 ng/ml)                | (102 ng/ml)           | (9.64 ng/ml)              | (96.5 ng/ml)          |
| Cycle                   |                             |                       |                           |                       |
| 1                       | 10.9                        | 103                   | 9.89                      | 94.3                  |
| 2                       | 10.8                        | 103                   | 9.73                      | 95.6                  |
| 3                       | 10.6                        | 102                   | 9.68                      | 94.4                  |
| 4                       | 11.6                        | 112                   | 9.03                      | 98.8                  |
| 5                       | 11.1                        | 102                   | 8.93                      | 93.0                  |

**TABLE 11B: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON HALOFANTRINE AND WR 178,460 (AS FREE BASES) SPIKED HUMAN BLOOD SAMPLES<sup>@ ¥</sup>**

| Spiked<br>Concentration | HALOFANTRINE<br>(free base) |                       | WR 178,460<br>(free base) |                       |
|-------------------------|-----------------------------|-----------------------|---------------------------|-----------------------|
|                         | Low<br>Concentration        | High<br>Concentration | Low<br>Concentration      | High<br>Concentration |
|                         | (10.2 ng/ml)                | (102 ng/ml)           | (9.64 ng/ml)              | (96.5 ng/ml)          |
| Cycle                   |                             |                       |                           |                       |
| 1                       | 10.4                        | 90.4                  | 9.24                      | 89.1                  |
| 2                       | 10.1                        | 93.4                  | 9.35                      | 91.3                  |
| 3                       | 10.0                        | 95.0                  | 9.39                      | 89.9                  |
| 4                       | 9.74                        | 92.5                  | 9.27                      | 90.8                  |
| 5                       | 9.92                        | 99.4                  | 9.12                      | 92.6                  |

<sup>#</sup> Measured concentrations are averages of two analyses.

<sup>@</sup> Individually spiked samples.

<sup>¥</sup> Measured concentrations are averages of three analyses.

**TABLE 12A: ACCURACY OF HALOFANTRINE (FREE BASE) HUMAN BLOOD ASSAY (BLIND STUDY RESULTS)**

| Sample Number | Spiked Level (ng/ml) | Measured Level# (ng/ml) | Statistics (ng/ml)   |
|---------------|----------------------|-------------------------|----------------------|
| 1             | 0                    | *                       | Mean =               |
| 12            |                      | *                       | SD =                 |
| 13            |                      | *                       | Percent CV =         |
| 24            |                      | *                       | Percent Bias =       |
| 2             | 2.04                 | 1.82                    | Mean = 2.05          |
| 11            |                      | 2.05                    | SD = 0.170           |
| 15            |                      | 2.09                    | Percent CV = 8.31    |
| 22            |                      | 2.23                    | Percent Bias = 0.368 |
| 3             | 20.4                 | 20.0                    | Mean = 19.8          |
| 10            |                      | 20.0                    | SD = 0.300           |
| 14            |                      | 19.6                    | Percent CV = 1.52    |
| 23            |                      | 19.4                    | Percent Bias = -3.19 |
| 4             | 40.8                 | 40.8                    | Mean = 39.3          |
| 8             |                      | 36.5                    | SD = 2.19            |
| 17            |                      | 41.3                    | Percent CV = 5.58    |
| 21            |                      | 38.7                    | Percent Bias = -3.62 |
| 5             | 102                  | 90.9                    | Mean = 93.6          |
| 9             |                      | 92.9                    | SD = 2.24            |
| 16            |                      | 96.1                    | Percent CV = 2.39    |
| 20            |                      | 94.6                    | Percent Bias = -8.21 |
| 6             | 183.6                | 171                     | Mean = 173           |
| 7             |                      | 172                     | SD = 2.63            |
| 18            |                      | 173                     | Percent CV = 1.52    |
| 19            |                      | 177                     | Percent Bias = -5.64 |

# Measured concentrations are averages of three analyses.

\* = Below assay sensitivity.

**TABLE 12B: ACCURACY OF WR 178,460 (FREE BASE) HUMAN BLOOD ASSAY (BLIND STUDY RESULTS)**

| Sample Number | Spiked Level (ng/ml) | Measured Level# (ng/ml) | Statistics (ng/ml)    |
|---------------|----------------------|-------------------------|-----------------------|
| 6             | 0                    | 0.893                   | Mean = 1.03           |
| 7             |                      | 1.05                    | SD = 0.121            |
| 18            |                      | 0.982                   | Percent CV = 11.8     |
| 19            |                      | 1.18                    | Percent Bias =        |
| 5             | 1.97                 | 2.52                    | Mean = 2.635          |
| 9             |                      | 2.50                    | SD = 0.150            |
| 16            |                      | 2.71                    | Percent CV = 5.70     |
| 20            |                      | 2.81                    | Percent Bias = 33.8   |
| 4             | 19.7                 | 20.2                    | Mean = 19.8           |
| 8             |                      | 19.3                    | SD = 0.45             |
| 17            |                      | 19.6                    | Percent CV = 2.27     |
| 21            |                      | 20.2                    | Percent Bias = 0.635  |
| 3             | 39.4                 | 38.2                    | Mean = 38.4           |
| 10            |                      | 37.6                    | SD = 0.665            |
| 14            |                      | 38.8                    | Percent CV = 1.73     |
| 23            |                      | 39.1                    | Percent Bias = -2.47  |
| 2             | 98.6                 | 95.2                    | Mean = 98.3           |
| 11            |                      | 96.3                    | SD = 3.12             |
| 15            |                      | 102                     | Percent CV = 3.18     |
| 22            |                      | 99.7                    | Percent Bias = -0.304 |
| 1             | 177.5                | 178                     | Mean = 181            |
| 12            |                      | 178                     | SD = 3.79             |
| 13            |                      | 186                     | Percent CV = 2.10     |
| 24            |                      | 180                     | Percent Bias = 1.69   |

# Measured concentrations are averages of three analyses.

**LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460  
AS FREE BASES IN RAT PERFUSATE PRECIPITATION ASSAY,\* STUDY  
REPORT 17B, SUPPLEMENT I**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BB43807, expiration date not available.
4. WR 178,460 (hydrochloride) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK21070, expiration date not available.
5. WR 122,455 (internal standard) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. AX26839, expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

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\* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

### C. ASSAY CONDITIONS

#### 1. DETECTOR

##### Settings

Wavelength: excitation - 300 nm, emission - 375 nm

Sensitivity: high

Range: 4

Response: medium

##### Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

#### 2. COLUMN

Axxiom Silica, 5  $\mu$ m particle size, 4.6 x 250 mm  
(Richard Scientific, Novato, CA).

#### 3. SOLVENT SYSTEM

Combine and mix H<sub>2</sub>O (800 mL) + (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (20 mL of 1 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) + CH<sub>3</sub>OH (3200 L).

#### 4. FLOW RATE

1.0 ml/min

5. STOCK SOLUTIONS - Solutions were stored in the xx°C freezer (refrigerator) and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

#### A. Stock Solutions

- i. HALOFANTRINE - (Halofantrine Hydrochloride free base concentrations).

Prep date: 3/1/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------------|
| Standard Curve | 5.60                    | 0.942          | 50.6           | methanol | 104                 |
| Precision      | 6.24                    | 0.942          | 50.0           | methanol | 118                 |

\*= Molecular weights of halofantrine free base/halofantrine hydrochloride

- ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride, free base concentrations).

Prep date: 3/1/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------|
| Standard Curve | 5.60                    | 0.924          | 50.6           | methanol | 102           |
| Precision      | 6.03                    | 0.924          | 52.0           | methanol | 107           |

\*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

- iii. WR 122,455 (Internal Standard) - (WRAIR, Washington, D.C.).

Prep date: 12/14/93

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|---------------|-------------------------|---------------|----------------|----------|---------------|
| Internal std. | 5.20                    | 1             | 25             | methanol | 208           |

## B. Working Solutions

- i. HALOFANTRINE AND WR 178,460 MIXED WORKING SOLUTIONS (free base concentrations).

- a. HIGH CONCENTRATION WORKING SOLUTION -

Combine 10.0 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions.

Prep date: 3/22/94

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 10.0                | 20.0           | methanol | 52.0          |
| Precision (Halofantrine)      | 118                   | 10.0                | 20.0           | methanol | 59.0          |
| Standard Curve (WR 178,460)   | 102                   | 10.0                | 20.0           | methanol | 51.0          |
| Precision (WR 178,460)        | 107                   | 10.0                | 20.0           | methanol | 53.5          |

## b. LOW CONCENTRATION WORKING SOLUTION -

Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 10 ml.

Prep date: 3/22/94

| Solution Type                    | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>(ng/ml) |
|----------------------------------|------------------------------------------|---------------------------|----------------------|----------|------------------|
| Standard Curve<br>(Halofantrine) | 104                                      | 1.00                      | 10.0                 | methanol | 10.4             |
| Precision<br>(Halofantrine)      | 118                                      | 1.00                      | 10.0                 | methanol | 11.8             |
| Standard Curve<br>(WR 178,460)   | 102                                      | 1.00                      | 10.0                 | methanol | 10.2             |
| Precision<br>(WR 178,460)        | 107                                      | 1.00                      | 10.0                 | methanol | 10.7             |

## ii. WR 122,455 - Internal standard.

Prep date: 3/22/94

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>( $\mu\text{g/ml}$ ) |
|---------------|------------------------------------------|---------------------------|----------------------|----------|-------------------------------|
| Internal std. | 208                                      | 0.500                     | 10.5                 | methanol | 9.90                          |

## 7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

- a. Halofantrine - 6.2 min
- b. WR 178,460 - 8.6 min
- c. WR 122,455 (Internal Standard) - 10.3 min

## 8. BLANK RAT PERFUSATE

Supplied by WRAIR.

## 9. INJECTION VOLUME

5-20  $\mu\text{l}$

## 10. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of  $1/y_i$ .

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat perfusate were based on the interday and intraday low point validation results (Table 2) and on standard curve calibrator results (Tables 1 and 4).)

5.20 µg/ml halofantrine (free base).

5.10 µg/ml WR 178,460 (free base).

12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

Plasma samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

13. WISP OPERATING TEMPERATURE

Room temperature.

#### D. SAMPLE STORAGE

All samples are to be kept frozen at -70°C before analysis and thawed at room temperature for preparation (within 30 min) and analysis.

#### E. SAMPLE PREPARATION

1. Pipet 100 µl rat perfusate samples into 13 X 100 silanized tubes.
2. Add 40 µl of 9.90 µg/ml WR 122,455 internal standard solution. Vortex for 30s.
3. Add 0.4 ml CH<sub>3</sub>CN. Vortex 1 min.
4. Centrifuge 10 min at 3000 g.
5. Transfer supernatant to silanized inserts and inject onto column.

#### F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 0.1 ml perfusate samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

### Generation of Halofantrine Standard Curve Samples

| Sample | Volume Spiked (µl) | Spiking Solution Concentration (µg/ml) | Mass Spiked (µg) | Standard Curve Sample Nominal Concentration (µg/ml) |
|--------|--------------------|----------------------------------------|------------------|-----------------------------------------------------|
| 00*    | 0                  | 10.4                                   | 0                | 0                                                   |
| 0**    | 0                  | 10.4                                   | 0                | 0                                                   |
| 1      | 5                  | 10.4                                   | 52.0             | 0.520                                               |
| 2      | 10                 | 10.4                                   | 104              | 1.04                                                |
| 3      | 20                 | 10.4                                   | 208              | 2.08                                                |
| 4      | 8                  | 52.0                                   | 416              | 4.16                                                |
| 5      | 16                 | 52.0                                   | 832              | 8.32                                                |
| 6      | 32                 | 52.0                                   | 1664             | 16.64                                               |
| 7      | 64                 | 52.0                                   | 3328             | 33.28                                               |

### Generation of WR 178,460 Standard Curve Samples

| Sample | Volume Spiked (µl) | Spiking Solution Concentration (µg/ml) | Mass Spiked (µg) | Standard Curve Sample Nominal Concentration (µg/ml) |
|--------|--------------------|----------------------------------------|------------------|-----------------------------------------------------|
| 00     | 0                  | 10.2                                   | 0                | 0                                                   |
| 0      | 0                  | 10.2                                   | 0                | 0                                                   |
| 1      | 5                  | 10.2                                   | 51.0             | 0.510                                               |
| 2      | 10                 | 10.2                                   | 102              | 1.02                                                |
| 3      | 20                 | 10.2                                   | 204              | 2.04                                                |
| 4      | 8                  | 51.0                                   | 408              | 4.08                                                |
| 5      | 16                 | 51.0                                   | 816              | 8.16                                                |
| 6      | 32                 | 51.0                                   | 1632             | 16.32                                               |
| 7      | 64                 | 51.0                                   | 3264             | 32.64                                               |

## G. QUALITY CONTROL

### 1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

### 2. Pipette Calibration

See ASOP 2C-1.1.

### 3. Balance Calibration

See ASOP 2C-2.1.

## H. RECOVERY

Assay recovery was assessed at three different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in perfusate samples. The perfusate samples were generated by

\* 00 = Sample with no drug and no internal standard.

\*\* 0 = Sample with no drug but with internal standard.

spiking 100  $\mu$ l of blank rat perfusate with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation) prior to addition of acetonitrile precipitant. Each perfusate sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added to the transferred supernatant (after step 5, not in step 3). The reference samples were generated by spiking water (100  $\mu$ l in silanized tubes) with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). Reference samples were prepared by vortexing in acetonitrile, then internal standard, and transferring to silanized inserts for injection onto the HPLC column.

## I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 100  $\mu$ l perfusate specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

### Generation of Halofantrine Precision Samples

|      | Volume Spiked ( $\mu$ l) | Spiking Solution Concentration ( $\mu$ g/ml) | Control Volume ( $\mu$ l) | Precision Sample Nominal Concentration ( $\mu$ g/ml) |
|------|--------------------------|----------------------------------------------|---------------------------|------------------------------------------------------|
| Low  | 10                       | 11.8                                         | 100                       | 1.18                                                 |
| Med. | 10                       | 59.0                                         | 100                       | 5.90                                                 |
| Hi   | 40                       | 59.0                                         | 100                       | 23.6                                                 |

### Generation of WR 178,460 Precision Samples

|      | Volume Spiked ( $\mu$ l) | Spiking Solution Concentration ( $\mu$ g/ml) | Control Volume ( $\mu$ l) | Precision Sample Nominal Concentration ( $\mu$ g/ml) |
|------|--------------------------|----------------------------------------------|---------------------------|------------------------------------------------------|
| Low  | 10                       | 10.7                                         | 100                       | 1.07                                                 |
| Med. | 10                       | 53.5                                         | 100                       | 5.35                                                 |
| Hi   | 40                       | 53.5                                         | 100                       | 20.4                                                 |

## J. RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

### 2. LOW POINT VALIDATION

Results for this evaluation appears in Table 2.

3. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 3.

3. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4A-D.

4. RECOVERY

Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
HALOFANTRINE (FREE BASE) RAT PERFUSATE  
PRECIPITATION ASSAY,  
STUDY REPORT 17B, SUPPLEMENT NO. 1

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(µg/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(µg/ml) |       |                           |                                        |
| 0                         | 0                        | 0     | -                         |                                        |
| 52.0                      | 0.520                    | 0.093 | 0.568                     |                                        |
| 104                       | 1.04                     | 0.172 | 0.992                     |                                        |
| 208                       | 2.08                     | 0.366 | 2.03                      |                                        |
| 416                       | 4.16                     | 0.751 | 4.10                      |                                        |
| 832                       | 8.32                     | 1.526 | 8.26                      |                                        |
| 1664                      | 16.64                    | 3.064 | 16.5                      |                                        |
| 3328                      | 33.28                    | 6.238 | 33.6                      |                                        |

\*\*\*

Regression equation:  
 $y = 0.186x - 0.0127, r^2 = 0.9998$

\* Into 100 µl of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y$ .

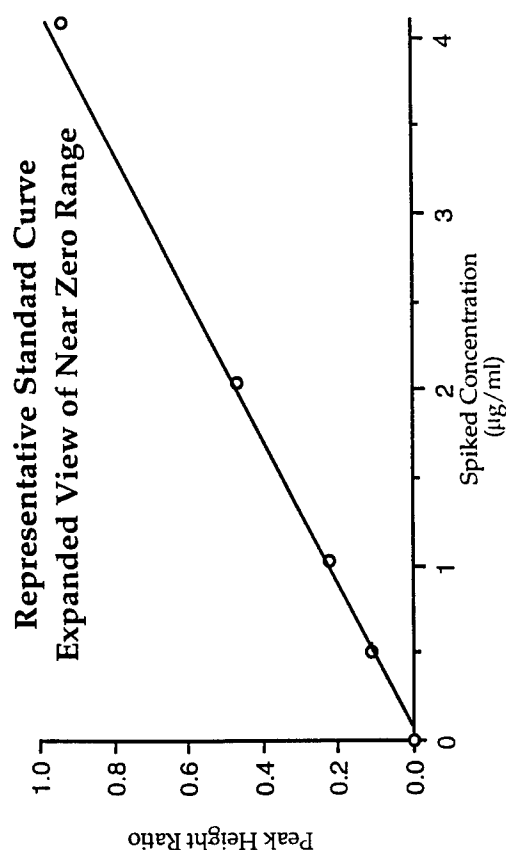
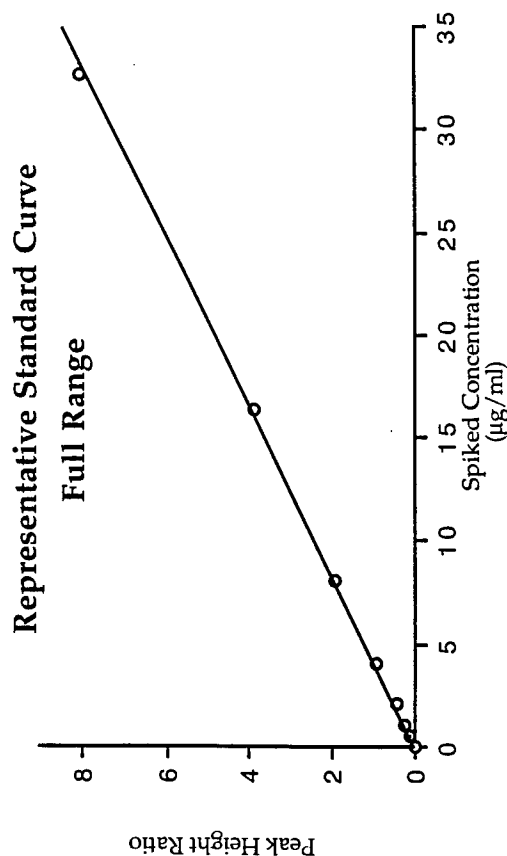


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
WR 178,460 (FREE BASE) RAT PERFUSATE  
PRECIPITATION ASSAY,  
STUDY REPORT 17B, SUPPLEMENT NO. I

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE                            |                                              | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>( $\mu\text{g}/\text{ml}$ ) |
|---------------------------|----------------------------------------------|----------------------------------------------|---------------------------|------------------------------------------------------------|
|                           | CONCENTRATION<br>( $\mu\text{g}/\text{ml}$ ) | CONCENTRATION<br>( $\mu\text{g}/\text{ml}$ ) |                           |                                                            |
| 0                         | 0                                            | 0                                            | 0                         | -                                                          |
| 51.0                      | 0.510                                        | 0.510                                        | 0.115                     | 0.553                                                      |
| 102                       | 1.02                                         | 1.02                                         | 0.224                     | 1.00                                                       |
| 204                       | 2.04                                         | 2.04                                         | 0.466                     | 2.00                                                       |
| 408                       | 4.08                                         | 4.08                                         | 0.939                     | 3.95                                                       |
| 816                       | 8.16                                         | 8.16                                         | 1.920                     | 7.99                                                       |
| 1632                      | 16.32                                        | 16.32                                        | 3.896                     | 16.1                                                       |
| 3264                      | 32.64                                        | 32.64                                        | 8.028                     | 33.2                                                       |

Regression equation:\*\*\*

$$y = 0.243x - 0.0192, \quad r^2 = 0.9996$$

\* Into 100  $\mu\text{l}$  of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

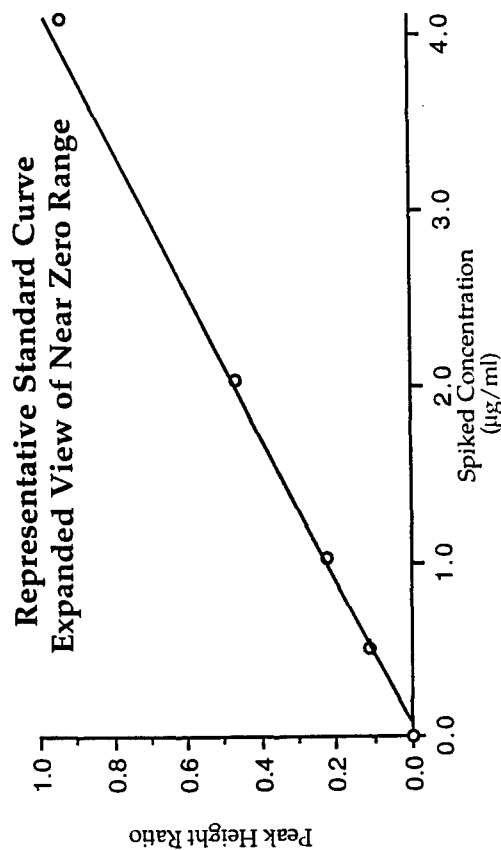
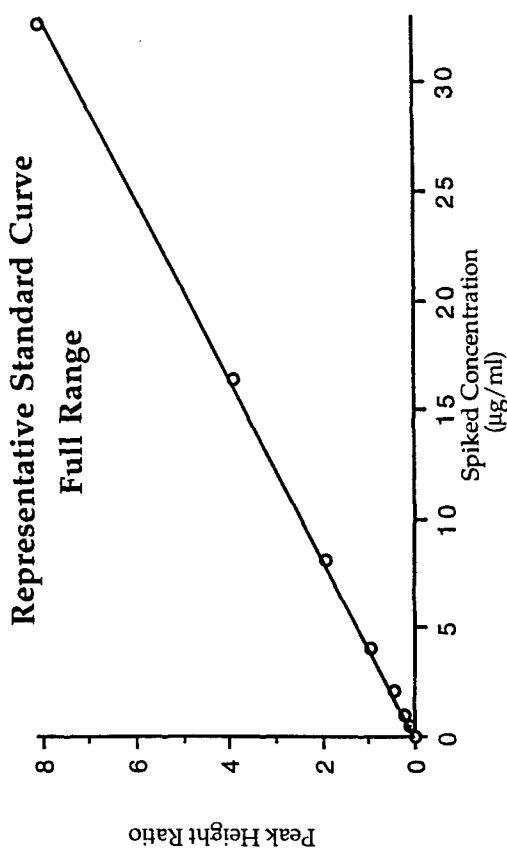


**TABLE 2: LOW POINT VALIDATION OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) RAT PERFUSATE  
PRECIPITATION ASSAY**

| Spiked Concentration | HALOFANTRINE<br>(free base)<br>(0.520 µg/ml) |          | WR 178,460<br>(free base)<br>(0.510 µg/ml) |          |
|----------------------|----------------------------------------------|----------|--------------------------------------------|----------|
|                      | Measured Concentrations<br>(µg/ml)           |          |                                            |          |
|                      | Interday                                     | Intraday | Interday                                   | Intraday |
|                      | 0.568                                        | 0.482    | 0.553                                      | 0.543    |
|                      | 0.545                                        | 0.459    | 0.554                                      | 0.561    |
|                      | 0.546                                        | 0.523    | 0.558                                      | 0.600    |
|                      |                                              | 0.593    |                                            | 0.517    |
|                      |                                              | 0.476    |                                            | 0.504    |
|                      |                                              | 0.476    |                                            | 0.622    |
| Mean                 | 0.553                                        | 0.502    | 0.555                                      | 0.558    |
| S. D.                | 0.013                                        | 0.050    | 0.003                                      | 0.046    |
| Percent C.V.         | 2.35                                         | 9.90     | 0.48                                       | 8.29     |
| Percent R.E.         | 6.35                                         | -3.56    | 8.82                                       | 9.38     |

**TABLE 3: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE  
AND WR 178,460 AS FREE BASES RAT PERFUSATE  
PRECIPITATE ASSAY, STUDY REPORT 17B, SUPPLEMENT I**

Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope       | Intercept    | Coefficient of<br>Determination |
|------------------------|----------------|-------------|--------------|---------------------------------|
| <u>Halofantrine</u>    |                |             |              |                                 |
| 4/7/94                 | 1(intraday)    | 0.18495382  | -0.00766061  | 0.999565216                     |
| 4/8/94                 | 2(interday 1)  | 0.186197073 | -0.012688465 | 0.999762579                     |
| 4/8/94                 | 3(interday 2)  | 0.186384551 | -0.018650289 | 0.99982947                      |
| 4/8/94                 | 4(Interday 3)  | 0.186839457 | -0.016978611 | 0.999406029                     |
| <u>WR 178460</u>       |                |             |              |                                 |
| 4/7/94                 | 1A(intraday)   | 0.242112571 | -0.021091236 | 0.99937132                      |
| 4/8/94                 | 2A(interday 1) | 0.242656365 | -0.019223641 | 0.999551985                     |
| 4/8/94                 | 3A(interday 2) | 0.242181938 | -0.026159153 | 0.999610049                     |
| 4/8/94                 | 4A(Interday 3) | 0.244046863 | -0.023255414 | 0.998919693                     |

Halofantrine Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |       |       |       |       |       |       |
|-------------------|---------------------------------------|-------|-------|-------|-------|-------|-------|
|                   | 0.52                                  | 1.04  | 2.08  | 4.16  | 8.32  | 16.64 | 33.28 |
|                   | Back Calculated Concentration (µg/ml) |       |       |       |       |       |       |
| 1                 | 0.571                                 | 1.01  | 2.03  | 4.07  | 8.09  | 16.5  | 33.8  |
| 2                 | 0.568                                 | 0.992 | 2.03  | 4.10  | 8.26  | 16.5  | 33.6  |
| 3                 | 0.545                                 | 1.02  | 2.02  | 4.12  | 8.29  | 16.9  | 33.1  |
| 4                 | 0.546                                 | 1.05  | 2.04  | 4.05  | 7.93  | 17.0  | 33.5  |
| Mean              | 0.558                                 | 1.02  | 2.03  | 4.09  | 8.14  | 16.7  | 33.5  |
| S.D.              | 0.014                                 | 0.024 | 0.008 | 0.031 | 0.167 | 0.263 | 0.294 |
| Percent C.V.      | 2.50                                  | 2.38  | 0.402 | 0.761 | 2.05  | 1.57  | 0.879 |
| Percent R.E.      | 7.21                                  | -2.12 | -2.40 | -1.80 | -2.13 | 0.511 | 0.661 |

WR 178460 Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |       |       |       |       |       |       |
|-------------------|---------------------------------------|-------|-------|-------|-------|-------|-------|
|                   | 0.510                                 | 1.02  | 2.04  | 4.08  | 8.16  | 16.32 | 32.64 |
|                   | Back Calculated Concentration (µg/ml) |       |       |       |       |       |       |
| 1A                | 0.554                                 | 1.01  | 2.01  | 3.94  | 7.86  | 16.2  | 33.2  |
| 2A                | 0.553                                 | 1.00  | 2.00  | 3.95  | 7.99  | 16.1  | 33.2  |
| 3A                | 0.554                                 | 1.00  | 1.96  | 3.96  | 7.95  | 16.5  | 32.8  |
| 4A                | 0.558                                 | 1.03  | 1.96  | 3.91  | 7.68  | 16.6  | 33.1  |
| Mean              | 0.555                                 | 1.01  | 1.98  | 3.94  | 7.87  | 16.4  | 33.1  |
| S.D.              | 0.002                                 | 0.014 | 0.026 | 0.022 | 0.138 | 0.238 | 0.189 |
| Percent C.V.      | 0.400                                 | 1.40  | 1.33  | 0.548 | 1.75  | 1.46  | 0.572 |
| Percent R.E.      | 8.77                                  | -0.98 | -2.82 | -3.43 | -3.55 | 0.184 | 1.33  |

**TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSION PRECIPITATION ASSAY**

Interday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/mL) |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|
|                                 |                  | 1.18                          | 5.90  | 23.6  |
| Measured Concentrations (µg/mL) |                  |                               |       |       |
| 2                               | 1                | 1.16                          | 5.49  | 23.1  |
|                                 | 2                | 1.20                          | 5.42  | 22.9  |
| 3                               | 1                | 1.18                          | 5.92  | 23.8  |
|                                 | 2                | 1.18                          | 5.91  | 23.9  |
| 4                               | 1                | 1.19                          | 5.46  | 22.4  |
|                                 | 2                | 1.16                          | 5.53  | 23.1  |
| Mean                            |                  | 1.18                          | 5.62  | 23.2  |
| S.D.                            |                  | 0.016                         | 0.23  | 0.566 |
| Percent C.V.                    |                  | 1.36                          | 4.09  | 2.44  |
| Percent R.E.                    |                  | -0.141                        | -4.72 | -1.69 |

**TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSION PRECIPITATION ASSAY**

Intraday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/mL) |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|
|                                 |                  | 1.18                          | 5.90  | 23.6  |
| Measured Concentrations (µg/mL) |                  |                               |       |       |
| 1                               | 1                | 1.10                          | 5.60  | 23.2  |
|                                 | 2                | 1.11                          | 5.83  | 23.0  |
|                                 | 3                | 1.09                          | 5.79  | 23.3  |
|                                 | 4                | 1.13                          | 5.49  | 22.7  |
|                                 | 5                | 1.17                          | 5.52  | 22.3  |
|                                 | 6                | 1.19                          | 5.30  | 23.0  |
| Mean                            |                  | 1.13                          | 5.59  | 22.9  |
| S.D.                            |                  | 0.040                         | 0.198 | 0.37  |
| Percent C.V.                    |                  | 3.55                          | 3.55  | 1.60  |
| Percent R.E.                    |                  | -4.10                         | -5.28 | -2.90 |

**TABLE 4C: PRECISION OF WR 178,460 FREE BASE RAT PERFUSION  
PRECIPITATION ASSAY**

Interday Precision WR 178460 (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/mL) |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|
|                                 |                  | 1.07                          | 5.35  | 20.4  |
| Measured Concentrations (µg/mL) |                  |                               |       |       |
| 2A                              | 1                | 1.02                          | 5.01  | 21.1  |
|                                 | 2                | 1.06                          | 4.99  | 21.2  |
| 3A                              | 1                | 1.12                          | 5.33  | 21.9  |
|                                 | 2                | 1.07                          | 5.31  | 21.8  |
| 4A                              | 1                | 1.06                          | 4.92  | 20.5  |
|                                 | 2                | 1.10                          | 4.96  | 21.4  |
| Mean                            |                  | 1.07                          | 5.09  | 21.3  |
| S.D.                            |                  | 0.0349                        | 0.183 | 0.512 |
| Percent C.V.                    |                  | 3.25                          | 3.61  | 2.40  |
| Percent R.E.                    |                  | 0.156                         | -4.92 | 4.49  |

**TABLE 4D: PRECISION OF WR 178,460 FREE BASE RAT PERFUSION  
PRECIPITATION ASSAY**

Intraday Precision WR 178460 (Free Base)

| Validation Run                  | QC Sample No. | Spiked Concentrations (µg/mL) |       |      |
|---------------------------------|---------------|-------------------------------|-------|------|
|                                 |               | 1.07                          | 5.35  | 20.4 |
| Measured Concentrations (µg/mL) |               |                               |       |      |
| 1A                              | 1             | 1.09                          | 5.08  | 21.5 |
|                                 | 2             | 1.07                          | 5.34  | 21.5 |
|                                 | 3             | 1.02                          | 5.22  | 21.4 |
|                                 | 4             | 1.06                          | 4.96  | 21.2 |
|                                 | 5             | 1.08                          | 5.02  | 20.4 |
|                                 | 6             | 1.07                          | 4.87  | 20.9 |
| Mean                            |               | 1.07                          | 5.08  | 21.2 |
| S.D.                            |               | 0.024                         | 0.173 | 0.43 |
| Percent C.V.                    |               | 2.28                          | 3.40  | 2.04 |
| Percent R.E.                    |               | -0.467                        | -5.02 | 3.68 |

**TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT PERFUSION BY PRECIPITATION**

| SAMPLE ID                  | SPIKED CONCENTRATION Range | SPIKED CONCENTRATION (µg/ml) | PEAK HEIGHT RATIO |               | MEAN PERCENT RECOVERY |
|----------------------------|----------------------------|------------------------------|-------------------|---------------|-----------------------|
|                            |                            |                              | REFERENCE         | PERFUSATE     |                       |
| <u>Halofantrine</u>        |                            |                              |                   |               |                       |
| 1                          | Low                        | 1.18                         | 0.100             | 0.101         | 101                   |
| 2                          |                            |                              | 0.104             | 0.122         |                       |
| 3                          |                            |                              | 0.120             | 0.105         |                       |
| Mean (± SD)                |                            |                              | 0.108 ± 0.011     | 0.109 ± 0.011 |                       |
| 1                          | Medium                     | 5.90                         | 0.611             | 0.596         | 100                   |
| 2                          |                            |                              | 0.607             | 0.609         |                       |
| 3                          |                            |                              | 0.575             | 0.590         |                       |
| Mean (± SD)                |                            |                              | 0.598 ± 0.020     | 0.598 ± 0.010 |                       |
| 1                          | High                       | 23.6                         | 2.457             | 2.255         | 96.0                  |
| 2                          |                            |                              | 2.354             | 2.363         |                       |
| 3                          |                            |                              | 2.413             | 2.314         |                       |
| Mean (± SD)                |                            |                              | 2.408 ± 0.052     | 2.311 ± 0.054 |                       |
| AVERAGE RECOVERY =         |                            |                              |                   |               | 99.1                  |
| <u>WR 178,460</u>          |                            |                              |                   |               |                       |
| 1                          | Low                        | 1.07                         | 0.122             | 0.124         | 106                   |
| 2                          |                            |                              | 0.119             | 0.140         |                       |
| 3                          |                            |                              | 0.138             | 0.138         |                       |
| Mean (± SD)                |                            |                              | 0.126 ± 0.010     | 0.134 ± 0.009 |                       |
| 1                          | Medium                     | 5.35                         | 0.696             | 0.698         | 101                   |
| 2                          |                            |                              | 0.684             | 0.701         |                       |
| 3                          |                            |                              | 0.669             | 0.678         |                       |
| Mean (± SD)                |                            |                              | 0.683 ± 0.014     | 0.692 ± 0.013 |                       |
| 1                          | High                       | 20.4                         | 2.843             | 2.575         | 93.6                  |
| 2                          |                            |                              | 2.773             | 2.657         |                       |
| 3                          |                            |                              | 2.800             | 2.649         |                       |
| Mean (± SD)                |                            |                              | 2.805 ± 0.035     | 2.627 ± 0.045 |                       |
| OVERALL AVERAGE RECOVERY = |                            |                              |                   |               | 100                   |

**LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460  
(AS FREE BASES) IN RAT PERFUSATE EXTRACTION ASSAY, STUDY  
REPORT 17, SUPPLEMENT II**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BB43807, expiration date not available.
4. WR 178,460 (hydrochloride) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BK21070, expiration date not available.
5. WR 122,455 (internal standard) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. AX26839, expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

## C. ASSAY CONDITIONS

### 1. DETECTOR

#### Settings

Wavelength: excitation - 300 nm, emission - 375 nm

Sensitivity: high

Range: 4

Response: medium

#### Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

### 2. COLUMN

Axxiom Silica, 5  $\mu$ m particle size, 4.6 x 250 mm  
(Richard Scientific, Novato, CA).

### 3. SOLVENT SYSTEM

Combine and mix H<sub>2</sub>O (800 mL) + (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (20 mL of 1 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) + CH<sub>3</sub>OH (3200 mL).

### 4. FLOW RATE

1.0 mL/min

5. STOCK SOLUTIONS - Solutions were stored in the 20°C freezer (refrigerator) and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (standard solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

#### A. Stock Solutions

- i. HALOFANTRINE - (Halofantrine Hydrochloride, WRAIR, Washington, D.C.), bottle number BB 43807.

Prep date: 3/1/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------------|
| Standard Curve | 5.60                    | 0.942          | 50.6           | methanol | 104                 |
| Precision      | 6.24                    | 0.942          | 50.0           | methanol | 118                 |

\*= Molecular weights of halofantrine free base/halofantrine hydrochloride

ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride, free base concentrations).

Prep date: 3/1/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------|
| Standard Curve | 5.60                    | 0.924          | 50.6           | methanol | 102           |
| Precision      | 6.03                    | 0.924          | 52.0           | methanol | 107           |

\*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

iii. WR 122,455 (Internal Standard) - (WRAIR, Washington, D.C.).

Prep date: 12/14/93

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|---------------|-------------------------|---------------|----------------|----------|---------------|
| Internal std. | 5.20                    | 1             | 25             | methanol | 208           |

B. Working Solutions

A. Halofantrine and WR 178,460 Mixed Working Solutions

1. High Concentration Working Solution: Combine 8.00 ml each of halofantrine and WR 178,460 (as free bases) stock solutions.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 8.00                | 100            | methanol | 8.32          |
| Precision (Halofantrine)      | 118                   | 8.00                | 100            | methanol | 9.44          |
| Standard Curve (WR 178,460)   | 102                   | 8.00                | 100            | methanol | 8.16          |
| Precision (WR 178,460)        | 107                   | 8.00                | 100            | methanol | 8.56          |

2. Low Concentration Working Solution: Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 1.00                | 100            | methanol | 1.04          |
| Precision (Halofantrine)      | 118                   | 1.00                | 100            | methanol | 1.18          |

| Solution Type                  | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>(ng/ml) |
|--------------------------------|------------------------------------------|---------------------------|----------------------|----------|------------------|
| Standard Curve<br>(WR 178,460) | 102                                      | 1.00                      | 100                  | methanol | 1.02             |
| Precision<br>(WR 178,460)      | 107                                      | 1.00                      | 100                  | methanol | 1.07             |

B. WR 122,455 - Internal standard.

Prep date: 3/4/94

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>( $\mu\text{g/ml}$ ) |
|---------------|------------------------------------------|---------------------------|----------------------|----------|-------------------------------|
| Internal std. | 208                                      | 0.5                       | 100                  | methanol | 1.04                          |

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

- Halofantrine - 7.4 min
- WR 178,460 - 11 min
- WR 122,455 (Internal Standard) - 13 min

8. BLANK RAT PERFUSATE

Supplied by WRAIR.

9. INJECTION VOLUME

50 - 100  $\mu\text{l}$

10. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of  $1/y_i$ .

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat perfusate were based on the interday and intraday low point validation results (Table 3) and on standard curve calibrator results (Tables 1 and 2).)

10.4 ng/ml halofantrine (free base).

10.2 ng/ml WR 178,460 (free base).

## 12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

Perfusate samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

## 13. WISP OPERATING TEMPERATURE

Room temperature.

### D. SAMPLE STORAGE

All samples are to be kept frozen at  $-20^{\circ}\text{C}$  before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

### E. SAMPLE PREPARATION

1. Pipet 100  $\mu\text{l}$  rat perfusate samples into 16 X 125 silanized tubes on ice. Add 100  $\mu\text{l}$  water.
2. Add 20  $\mu\text{l}$  of 1.04  $\mu\text{g}/\text{ml}$  WR 122,455 internal standard solution. Vortex for 30s.
3. Add 50  $\mu\text{l}$  0.1N NaOH. Vortex 30 s.
4. Add 2.0 ml methyl *t*-butyl ether. Vortex 1 min., twice. Centrifuge for 10 min. at 3000g.
5. Freeze aqueous layer in dry ice/methanol bath and pour organic layer into a 13 x 100 silanized tube.
6. Repeat steps 5 and 6.
7. Evaporate to dryness. Reconstitute in 4:1 methanol/water (v/v) with a final 0.001% HCl concentration.
8. Transfer supernatant to silanized inserts and inject onto column.

### F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 100  $\mu\text{l}$  perfusate samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

### Generation of Halofantrine Standard Curve Samples

| Sample | Volume Spiked (μl) | Spiking Solution Concentration (μg/ml) | Mass Spiked (ng) | Standard Curve Sample Nominal Concentration (ng/ml) |
|--------|--------------------|----------------------------------------|------------------|-----------------------------------------------------|
| 00     | 0                  | 0                                      | 0                | 0                                                   |
| 0      | 0                  | 0                                      | 0                | 0                                                   |
| 1      | 1                  | 1.04                                   | 1.04             | 10.4                                                |
| 2      | 2                  | 1.04                                   | 2.08             | 20.8                                                |
| 3      | 4                  | 1.04                                   | 4.16             | 41.6                                                |
| 4      | 8                  | 1.04                                   | 8.32             | 83.2                                                |
| 5      | 2                  | 8.32                                   | 16.64            | 166.4                                               |
| 6      | 4                  | 8.32                                   | 33.28            | 333                                                 |
| 7      | 8                  | 8.32                                   | 66.56            | 666                                                 |
| 8      | 16                 | 8.32                                   | 133.12           | 1331                                                |

### Generation of WR 178,460 Standard Curve Samples

| Sample | Volume Spiked (μl) | Spiking Solution Concentration (μg/ml) | Mass Spiked (ng) | Standard Curve Sample Nominal Concentration (ng/ml) |
|--------|--------------------|----------------------------------------|------------------|-----------------------------------------------------|
| 00     | 0                  | 0                                      | 0                | 0                                                   |
| 0      | 0                  | 0                                      | 0                | 0                                                   |
| 1      | 1                  | 1.02                                   | 1.02             | 10.2                                                |
| 2      | 2                  | 1.02                                   | 2.04             | 20.4                                                |
| 3      | 4                  | 1.02                                   | 4.08             | 40.8                                                |
| 4      | 8                  | 1.02                                   | 8.16             | 81.6                                                |
| 5      | 2                  | 8.16                                   | 16.32            | 163                                                 |
| 6      | 4                  | 8.16                                   | 32.64            | 326                                                 |
| 7      | 8                  | 8.16                                   | 65.28            | 653                                                 |
| 8      | 16                 | 8.16                                   | 130.56           | 1306                                                |

## G. QUALITY CONTROL

### 1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

### 2. Pipette Calibration

See ASOP 2C-1.1.

### 3. Balance Calibration

See ASOP 2C-2.1.

## H. RECOVERY

Assay recovery was assessed at four different halofantrine and WR 178,460 (as free bases) concentrations (equal to precision sample nominal concentrations) by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in perfusate (100  $\mu$ l) samples. The perfusate samples were generated by spiking on ice 100  $\mu$ l of blank rat perfusate to which water (100  $\mu$ l) has been added with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). Each perfusate sample was prepared as described in "Sample Preparation" above, except the internal standard was added to the transferred organic layer prior to evaporation. The reference samples (100  $\mu$ l rat perfusate) were generated by spiking methyl *t*-butyl ether extracts of rat perfusate (obtained as described in "Sample Preparation" above) with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). These reference samples were then prepared by completion of the sample preparation procedure as described (addition of IS, evaporation and reconstitution).

## I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 100  $\mu$ l perfusate specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

### Generation of Halofantrine Precision Samples

|      | Volume Spiked ( $\mu$ l) | Spiking Solution Concentration ( $\mu$ g/ml) | Control Volume ( $\mu$ l) | Precision Sample Nominal Concentration (ng/ml) |
|------|--------------------------|----------------------------------------------|---------------------------|------------------------------------------------|
| XL   | 2                        | 1.18                                         | 100                       | 23.6                                           |
| Low  | 6                        | 1.18                                         | 100                       | 70.8                                           |
| Med. | 3                        | 9.44                                         | 100                       | 283.2                                          |
| Hi   | 10                       | 9.44                                         | 100                       | 944                                            |

### Generation of WR 178,460 Precision Samples

|      | Volume Spiked ( $\mu$ l) | Spiking Solution Concentration ( $\mu$ g/ml) | Control Volume ( $\mu$ l) | Precision Sample Nominal Concentration (ng/ml) |
|------|--------------------------|----------------------------------------------|---------------------------|------------------------------------------------|
| XL   | 2                        | 1.07                                         | 100                       | 21.4                                           |
| Low  | 6                        | 1.07                                         | 100                       | 64.2                                           |
| Med. | 3                        | 8.56                                         | 100                       | 257                                            |
| Hi   | 10                       | 8.56                                         | 100                       | 856                                            |

## J. RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

### 2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 2.

### 3. LOW POINT VALIDATION

Results for this evaluation appears in Table 3.

### 4. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4A-D.

### 5. RECOVERY

Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
HALOFANTRINE (FREE BASE) RAT PERFUSATE  
EXTRACTION ASSAY,  
STUDY REPORT 17, SUPPLEMENT NO. 2

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) |       |                           |                                        |
| 0                         | 0                        | 0     | -                         |                                        |
| 1.04                      | 10.4                     | 0.046 | 11.5                      |                                        |
| 2.08                      | 20.8                     | 0.073 | 18.7                      |                                        |
| 4.16                      | 41.6                     | 0.163 | 42.6                      |                                        |
| 8.32                      | 83.2                     | 0.312 | 82.3                      |                                        |
| 16.64                     | 166.4                    | 0.625 | 166                       |                                        |
| 33.28                     | 333                      | 1.269 | 337                       |                                        |
| 66.56                     | 666                      | 2.496 | 663                       |                                        |
| 133.12                    | 1331                     | 5.009 | 1330                      |                                        |

Regression equation:\*\*\*

$$y = 0.00376x + 0.00269, \quad r^2 = 0.9998$$

\* Into 100  $\mu$ l of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

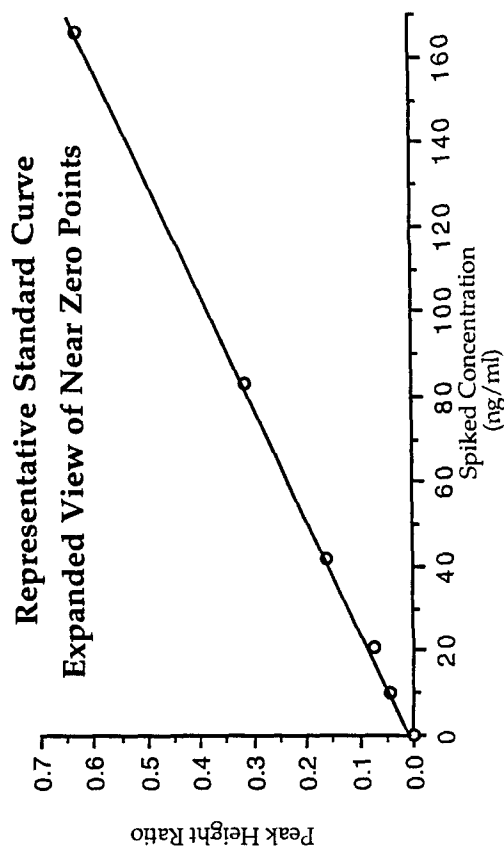
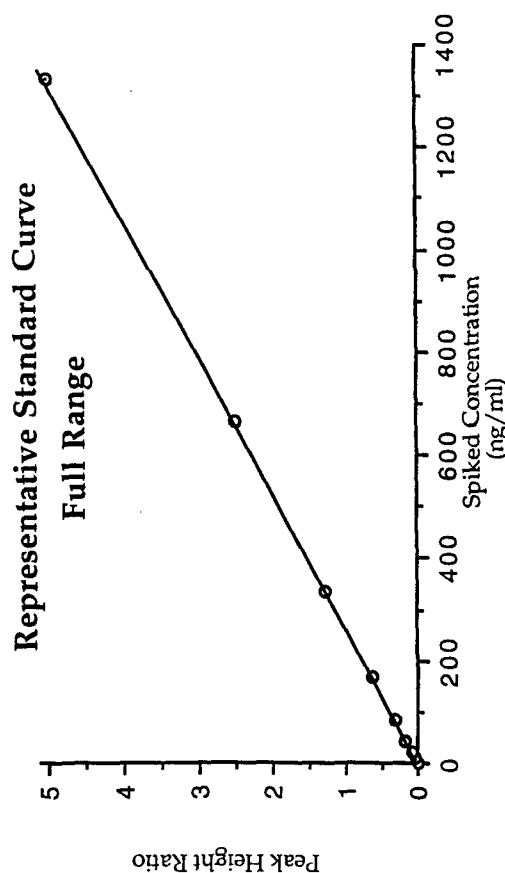


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
WR 178,460 (FREE BASE) RAT PERFUSATE  
EXTRACTION ASSAY,  
STUDY REPORT 17, SUPPLEMENT NO. 2

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |                          | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION |                          |
|---------------------------|--------------------------|--------------------------|---------------------------|-----------------------------|--------------------------|
|                           | CONCENTRATION<br>(ng/ml) | CONCENTRATION<br>(ng/ml) |                           | CONCENTRATION<br>(ng/ml)    | CONCENTRATION<br>(ng/ml) |
| 0                         | 0                        | 0                        | 0                         | -                           | -                        |
| 1.02                      | 10.2                     | 10.2                     | 0.042                     | 9.22                        | 9.22                     |
| 2.04                      | 20.4                     | 20.4                     | 0.116                     | 23.2                        | 23.2                     |
| 4.08                      | 40.8                     | 40.8                     | 0.235                     | 45.6                        | 45.6                     |
| 8.16                      | 81.6                     | 81.6                     | 0.414                     | 79.4                        | 79.4                     |
| 16.32                     | 163                      | 163                      | 0.859                     | 163                         | 163                      |
| 32.64                     | 326                      | 326                      | 1.583                     | 300                         | 300                      |
| 65.28                     | 653                      | 653                      | 3.346                     | 632                         | 632                      |
| 130.56                    | 1306                     | 1306                     | 7.174                     | 1350                        | 1350                     |

Regression equation:  
 $y = 0.00530x - 0.00691$ ,  $r^2 = 0.9975$

\* Into 100  $\mu$ l of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

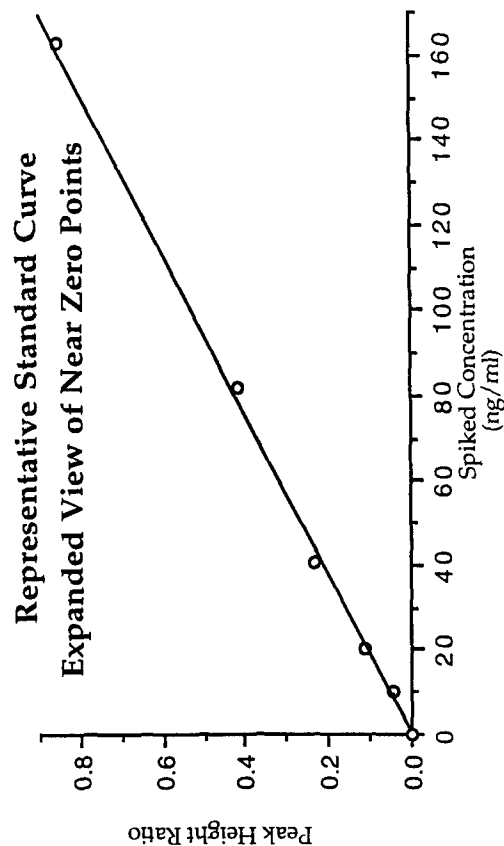
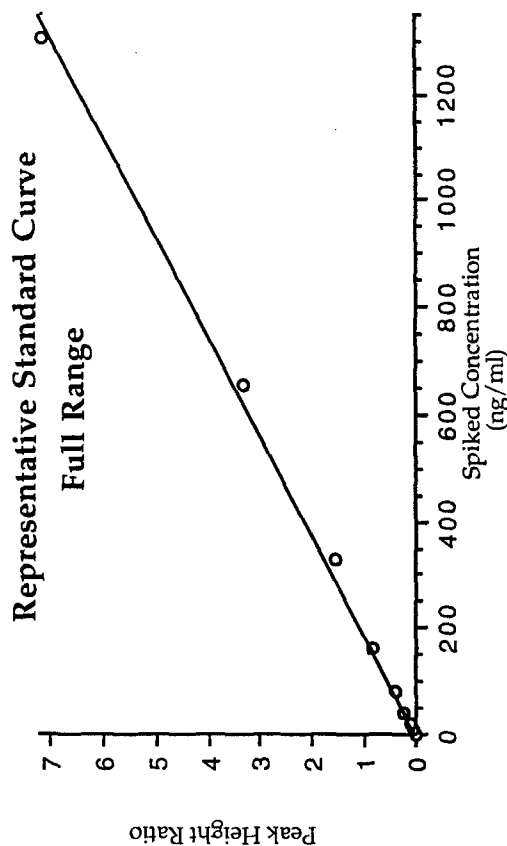


**TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE  
AND WR 178,460 AS FREE BASES RAT PERFUSATE  
EXTRACTION ASSAY, STUDY REPORT 17B, SUPPLEMENT II**

Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope       | Intercept    | Coefficient of<br>Determination |
|------------------------|----------------|-------------|--------------|---------------------------------|
| <u>Halofantrine</u>    |                |             |              |                                 |
| 5/27/94                | 1(interday 1)  | 0.003758804 | 0.002694172  | 0.999815534                     |
| 6/2/96                 | 2(intraday)    | 0.003635292 | -0.008268307 | 0.997896349                     |
| 6/2/96                 | 3(interday 2)  | 0.003507602 | 0.003106297  | 0.999308123                     |
| 6/18/94                | 4(interday 3)  | 0.004056372 | 0.007003992  | 0.999050706                     |
| <u>WR 178460</u>       |                |             |              |                                 |
| 5/27/94                | 1A(interday 1) | 0.005303248 | -0.006905876 | 0.997504309                     |
| 6/2/96                 | 2A(intraday)   | 0.005186956 | 0.008207105  | 0.998102803                     |
| 6/2/96                 | 3A(interday 2) | 0.005065837 | 0.019508885  | 0.999760483                     |
| 6/18/94                | 4A(interday 3) | 0.00529495  | 0.001261078  | 0.999771288                     |

Halofantrine Back Calculated Standard Calibrators

| Validation<br>Run                     | Spiked Concentration (ng/ml) |       |       |       |       |      |       |       |
|---------------------------------------|------------------------------|-------|-------|-------|-------|------|-------|-------|
|                                       | 10.4                         | 20.8  | 41.6  | 83.2  | 166.4 | 333  | 666   | 1331  |
| Back Calculated Concentration (ng/ml) |                              |       |       |       |       |      |       |       |
| 1                                     | 11.5                         | 18.7  | 42.6  | 82.3  | 166   | 337  | 663   | 1330  |
| 2                                     | 11.7                         | 19.1  | 38.2  | 83.2  | 176   | 341  | 671   | 1310  |
| 3                                     | 10.4                         | 20.7  | 45.4  | 75.9  | 163   | 352  | 659   | 1330  |
| 4                                     | 12.7                         | 16.9  | 39.4  | 83.7  | 176   | 361  | 652   | 1320  |
| Mean                                  | 11.6                         | 18.9  | 41.4  | 81.3  | 170   | 348  | 661   | 1323  |
| S.D.                                  | 0.943                        | 1.56  | 3.25  | 3.63  | 6.75  | 10.9 | 7.93  | 9.57  |
| Percent C.V.                          | 8.15                         | 8.28  | 7.85  | 4.47  | 3.97  | 3.13 | 1.20  | 0.72  |
| Percent R.E.                          | 11.3                         | -9.38 | -0.48 | -2.31 | 2.31  | 4.43 | -0.71 | -0.64 |

WR 178460 Back Calculated Standard Calibrators

| Validation<br>Run                     | Spiked Concentration (ng/ml) |      |      |       |      |       |       |      |
|---------------------------------------|------------------------------|------|------|-------|------|-------|-------|------|
|                                       | 10.2                         | 20.4 | 40.8 | 81.6  | 163  | 326   | 653   | 1306 |
| Back Calculated Concentration (ng/ml) |                              |      |      |       |      |       |       |      |
| 1A                                    | 9.22                         | 23.2 | 45.6 | 79.4  | 163  | 300   | 632   | 1350 |
| 2A                                    | 11.5                         | 20.2 | 39.2 | 80    | 162  | 318   | 654   | 1320 |
| 3A                                    | 10.3                         | 21.3 | 38.5 | 81.7  | 163  | 331   | 641   | 1310 |
| 4A                                    | 11.7                         | 19.2 | 42.2 | 72.6  | 176  | 318   | 628   | 1340 |
| Mean                                  | 10.7                         | 21.0 | 41.4 | 78.4  | 166  | 317   | 639   | 1330 |
| S.D.                                  | 1.15                         | 1.71 | 3.24 | 4.00  | 6.68 | 12.7  | 11.5  | 18.3 |
| Percent C.V.                          | 10.8                         | 8.17 | 7.84 | 5.11  | 4.03 | 4.02  | 1.80  | 1.37 |
| Percent R.E.                          | 4.71                         | 2.82 | 1.41 | -3.89 | 1.84 | -2.84 | -2.18 | 1.84 |

**TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) RAT PERFUSATE  
EXTRACTION ASSAY**

| Spiked Concentration    | HALOFANTRINE |          | WR 178,460   |       |
|-------------------------|--------------|----------|--------------|-------|
|                         | (free base)  |          | (free base)  |       |
|                         | (10.4 ng/ml) |          | (10.2 ng/ml) |       |
| Measured Concentrations |              |          |              |       |
| (ng/ml)                 |              |          |              |       |
| Interday                | Intraday     | Interday | Intraday     |       |
| 11.7                    | 12.5         | 9.28     | 10.1         |       |
| 11.7                    | 11.0         | 11.5     | 9.52         |       |
| 10.4                    | 10.4         | 10.3     | 10.4         |       |
|                         | 11.7         |          | 11.6         |       |
|                         | 11.2         |          | 10.8         |       |
|                         | 11.7         |          | 11.6         |       |
| Mean                    | 11.3         | 11.4     | 10.4         | 10.7  |
| Standard Deviation      | 0.751        | 0.719    | 1.11         | 0.833 |
| Percent C.V.            | 6.66         | 6.30     | 10.7         | 7.80  |
| Percent R.E.            | 8.33         | 9.78     | 1.57         | 4.61  |

**TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSATE EXTRACTION ASSAY**

Interday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (ng/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 23.6                          | 70.8  | 283.2 | 944   |
| Measured Concentrations (ng/ml) |                  |                               |       |       |       |
| 1                               | 1                | 21.6                          | 65.3  | 278   | 845   |
|                                 | 2                | 18.7                          | 67.1  | 261   | 851   |
| 3                               | 1                | 21.4                          | 70.4  | 280   | 935   |
|                                 | 2                | 23.3                          | 61.3  | 286   | 940   |
| 4                               | 1                | 21.4                          | 66.3  | 262   | 888   |
|                                 | 2                | bc                            | 72.7  | 298   | 912   |
| Mean                            |                  | 21.3                          | 67.2  | 278   | 895   |
| S.D.                            |                  | 1.65                          | 3.99  | 14.2  | 41    |
| Percent C.V.                    |                  | 7.75                          | 5.94  | 5.13  | 4.58  |
| Percent R.E.                    |                  | -9.83                         | -5.11 | -2.01 | -5.17 |

**TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT PERFUSATE EXTRACTION ASSAY**

Intraday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (ng/ml) |      |       |       |
|---------------------------------|------------------|-------------------------------|------|-------|-------|
|                                 |                  | 23.6                          | 70.8 | 283.2 | 944   |
| Measured Concentrations (ng/ml) |                  |                               |      |       |       |
| 2                               | 1                | 24.0                          | 73.0 | 255   | 919   |
|                                 | 2                | 21.3                          | 67.5 | 272   | 903   |
|                                 | 3                | 24.3                          | 72.1 | 270   | 917   |
|                                 | 4                | 25.7                          | 70.2 | 265   | 909   |
|                                 | 5                | 21.8                          | 72.1 | 278   | 912   |
|                                 | 6                | 24.3                          | 70.5 | 262   | 882   |
| Mean                            |                  | 23.6                          | 70.9 | 267   | 907   |
| S.D.                            |                  | 1.68                          | 1.97 | 8.10  | 13.5  |
| Percent C.V.                    |                  | 7.12                          | 2.79 | 3.03  | 1.49  |
| Percent R.E.                    |                  | -0.14                         | 0.14 | -5.72 | -3.92 |

**TABLE 4C: INTERDAY PRECISION OF WR 178,460 FREE BASE RAT  
PERFUSATE EXTRACTION ASSAY**

Interday Precision WR 178,460 (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (ng/ml) |       |      |       |
|---------------------------------|------------------|-------------------------------|-------|------|-------|
|                                 |                  | 21.4                          | 64.2  | 257  | 856   |
| Measured Concentrations (ng/ml) |                  |                               |       |      |       |
| 1A                              | 1                | 24.1                          | 62    | 257  | 780   |
|                                 | 2                | 22.2                          | 60.3  | 237  | 776   |
| 3A                              | 1                | 20.4                          | 64.3  | 257  | 869   |
|                                 | 2                | 22.4                          | 66    | 264  | 851   |
| 4A                              | 1                | 19.8                          | 59.4  | 245  | 807   |
|                                 | 2                | bc                            | 60.6  | 265  | 842   |
| Mean                            |                  | 21.8                          | 62.1  | 254  | 821   |
| S.D.                            |                  | 1.72                          | 2.56  | 11   | 38.9  |
| Percent C.V.                    |                  | 7.88                          | 4.13  | 4.34 | 4.73  |
| Percent R.E.                    |                  | 1.78                          | -3.27 | -1.1 | -4.11 |

**TABLE 4D: INTRADAY PRECISION OF WR 178,460 FREE BASE RAT  
PERFUSATE EXTRACTION ASSAY**

Intraday Precision WR 178,460 (Free Base)

| Validation Run                  | QC Sample No. | Spiked Concentrations (ng/ml) |       |       |       |
|---------------------------------|---------------|-------------------------------|-------|-------|-------|
|                                 |               | 21.4                          | 64.2  | 257   | 856   |
| Measured Concentrations (ng/ml) |               |                               |       |       |       |
| 2A                              | 1             | 21.7                          | 64.0  | 234   | 846   |
|                                 | 2             | 22.3                          | 69.6  | 252   | 834   |
|                                 | 3             | 23.7                          | 62.2  | 244   | 851   |
|                                 | 4             | 24.1                          | 53.7  | 243   | 843   |
|                                 | 5             | 20.6                          | 59.3  | 252   | 840   |
|                                 | 6             | 21.6                          | 57.8  | 233   | 844   |
| Mean                            |               | 22.3                          | 61.1  | 243   | 843   |
| S.D.                            |               | 1.34                          | 5.49  | 8.29  | 5.73  |
| Percent C.V.                    |               | 5.99                          | 8.99  | 3.41  | 0.68  |
| Percent R.E.                    |               | 4.36                          | -4.83 | -5.45 | -1.52 |

**TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT PERFUSATE BY EXTRACTION**

| SAMPLE ID                           | SPIKED CONCENTRATION<br>Range (µg/ml) |       | PEAK HEIGHT RATIO |              | MEAN PERCENT RECOVERY |
|-------------------------------------|---------------------------------------|-------|-------------------|--------------|-----------------------|
|                                     |                                       |       | REFERENCE         | PERFUSATE    |                       |
| <u>Halofantrine</u>                 |                                       |       |                   |              |                       |
| 1                                   | Extra Low                             | 23.6  | 0.115             | 0.100        | 84.2                  |
| 2                                   |                                       |       | 0.119             | 0.096        |                       |
| 3                                   |                                       |       | 0.114             | 0.097        |                       |
| Mean (± SD)                         |                                       |       | 0.116 ±0.003      | 0.098 ±0.002 |                       |
| 1                                   | Low                                   | 70.8  | 0.294             | 0.327        | 95.5                  |
| 2                                   |                                       |       | 0.287             | 0.298        |                       |
| 3                                   |                                       |       | 0.358             | 0.272        |                       |
| Mean (± SD)                         |                                       |       | 0.313 ±0.039      | 0.299 ±0.028 |                       |
| 1                                   | Medium                                | 283.2 | 1.143             | 1.085        | 91.9                  |
| 2                                   |                                       |       | 1.164             | 1.055        |                       |
| 3                                   |                                       |       | 1.199             | 1.081        |                       |
| Mean (± SD)                         |                                       |       | 1.169 ±0.028      | 1.074 ±0.016 |                       |
| 1                                   | High                                  | 944   | 3.426             | 3.624        | 91.7                  |
| 2                                   |                                       |       | 3.811             | 3.153        |                       |
| 3                                   |                                       |       | 3.756             | 3.306        |                       |
| Mean (± SD)                         |                                       |       | 3.664 ±0.208      | 3.361 ±0.240 |                       |
| <u>AVERAGE MEAN RECOVERY = 90.8</u> |                                       |       |                   |              |                       |
| <u>WR 178,460</u>                   |                                       |       |                   |              |                       |
| 1                                   | Extra Low                             | 21.4  | 0.116             | 0.111        | 91.3                  |
| 2                                   |                                       |       | 0.132             | 0.108        |                       |
| 3                                   |                                       |       | 0.118             | 0.115        |                       |
| Mean (± SD)                         |                                       |       | 0.122 ±0.009      | 0.111 ±0.004 |                       |
| 1                                   | Low                                   | 64.2  | 0.334             | 0.317        | 91.8                  |
| 2                                   |                                       |       | 0.371             | 0.317        |                       |
| 3                                   |                                       |       | 0.354             | 0.338        |                       |
| Mean (± SD)                         |                                       |       | 0.353 ±0.019      | 0.324 ±0.012 |                       |
| 1                                   | Medium                                | 257   | 1.369             | 1.314        | 93.2                  |
| 2                                   |                                       |       | 1.341             | 1.236        |                       |
| 3                                   |                                       |       | 1.415             | 1.295        |                       |
| Mean (± SD)                         |                                       |       | 1.375 ±0.037      | 1.282 ±0.041 |                       |
| 1                                   | High                                  | 856   | 4.066             | 4.372        | 93.3                  |
| 2                                   |                                       |       | 4.488             | 3.699        |                       |
| 3                                   |                                       |       | 4.486             | 4.099        |                       |
| Mean (± SD)                         |                                       |       | 4.347 ±0.243      | 4.057 ±0.338 |                       |
| <u>AVERAGE MEAN RECOVERY = 92.4</u> |                                       |       |                   |              |                       |

**LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460  
AS FREE BASES IN RAT BILE PRECIPITATION ASSAY, STUDY REPORT 17,  
SUPPLEMENT III**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
4. WR 178,460 (hydrochloride), bottle no. BK 21070, (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
5. WR 122,455 (internal standard), bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

## C. ASSAY CONDITIONS

### 1. DETECTOR

#### Settings

Wavelength: excitation - 300 nm, emission - 375 nm

Sensitivity: high

Range: 4

Response: medium

#### Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

### 2. COLUMN

Axxiom Silica, 5  $\mu$ m particle size, 4.6 x 250 mm  
(Richard Scientific, Novato, CA).

### 3. SOLVENT SYSTEM

Combine and mix H<sub>2</sub>O (800 ml) + (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (20 ml of 1 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) + CH<sub>3</sub>OH (3200 ml).

### 4. FLOW RATE

1.0 ml/min.

5. STOCK SOLUTIONS - Solutions were stored in the -20°C freezer (refrigerator) and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

#### A. Stock Solutions

##### i. HALOFANTRINE - (Halofantrine Hydrochloride).

| Prep date: 3/1/94 |                         |                |                |          |                     |
|-------------------|-------------------------|----------------|----------------|----------|---------------------|
| Solution Type     | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
| Standard Curve    | 5.60                    | 0.942          | 50.6           | methanol | 104                 |
| Precision         | 6.24                    | 0.942          | 50.0           | methanol | 118                 |

\*= Molecular weights of halofantrine free base/halofantrine hydrochloride

## ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride).

Prep date: 3/1/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------|
| Standard Curve | 5.60                    | 0.924          | 50.6           | methanol | 102           |
| Precision      | 6.03                    | 0.924          | 52.0           | methanol | 107           |

\* = Molecular weights of WR 178,460 free base / WR 178,460 hydrochloride

## iii. WR 122,455 (Internal Standard).

Prep date: 3/4/94

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|---------------|-------------------------|---------------|----------------|----------|---------------|
| Internal std. | 5.20                    | 1             | 25             | methanol | 208           |

## B. Working Solutions

## i. Halofantrine and WR 178,460 Mixed Working Solutions

- a. HIGH CONCENTRATION WORKING SOLUTION -  
Combine 10.0 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 10.0                | 20.0           | methanol | 52.0          |
| Standard Curve (WR 178,460)   | 102                   | 10.0                | 20.0           | methanol | 51.0          |
| Precision (Halofantrine)      | 118                   | 10.0                | 20.0           | methanol | 59.0          |
| Precision (WR 178,460)        | 107                   | 10.0                | 20.0           | methanol | 53.5          |

- b. LOW CONCENTRATION WORKING SOLUTION -  
Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 10 ml.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 1.00                | 10.0           | methanol | 10.4          |
| Standard Curve (WR 178,460)   | 102                   | 1.00                | 10.0           | methanol | 10.2          |

| Solution Type               | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>(ng/ml) |
|-----------------------------|------------------------------------------|---------------------------|----------------------|----------|------------------|
| Precision<br>(Halofantrine) | 118                                      | 1.00                      | 10.0                 | methanol | 11.8             |
| Precision<br>(WR 178,460)   | 107                                      | 1.00                      | 10.0                 | methanol | 10.7             |

ii. WR 122,455 - Internal standard.

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(part) | QS<br>Volume<br>(part) | Solvent  | Conc.<br>( $\mu\text{g/ml}$ ) |
|---------------|------------------------------------------|-----------------------------|------------------------|----------|-------------------------------|
| Internal std. | 208                                      | 0.5                         | 10.5                   | methanol | 9.90                          |

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.
  - a. Halofantrine - 7.2 min.
  - b. WR 178,460 - 10.7 min.
  - c. WR 122,455 (Internal Standard) - 12.7 min.
8. BLANK RAT BILE  
Supplied by WRAIR.
9. INJECTION VOLUME  
20  $\mu\text{l}$
10. QUANTITATION  
By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of  $1/y_i$ .
11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat bile were based on the interday and intraday low point validation results (Table 3).)  
0.416  $\mu\text{g/ml}$  halofantrine (free base).  
0.408  $\mu\text{g/ml}$  WR 178,460 (free base).
12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT  
Bile samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. Drugs are spiked with Hamilton syringes.

### 13. WISP OPERATING TEMPERATURE

Room temperature.

#### D. SAMPLE STORAGE

All samples are to be kept frozen at  $-70^{\circ}\text{C}$  before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

#### E. SAMPLE PREPARATION

1. Pipet 25  $\mu\text{l}$  rat bile samples into 13 X 100 silanized tubes.
2. Add 10  $\mu\text{l}$  of 9.90  $\mu\text{g}/\text{ml}$  WR 122,455 internal standard solution. Vortex for 30s.
3. Add 0.2 ml  $\text{CH}_3\text{CN}$ . Vortex 1 min.
4. Centrifuge at 3000 g for 10 min.
5. Transfer supernatant to silanized inserts and inject onto column.

#### F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 25  $\mu\text{l}$  bile samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

##### Generation of Halofantrine Standard Curve Samples

| Sample | Volume Spiked ( $\mu\text{l}$ ) | Spiking Solution Concentration ( $\mu\text{g}/\text{ml}$ ) | Mass Spiked ( $\mu\text{g}$ ) | Standard Curve Sample Nominal Concentration ( $\mu\text{g}/\text{ml}$ ) |
|--------|---------------------------------|------------------------------------------------------------|-------------------------------|-------------------------------------------------------------------------|
| 00     | 0                               | 10.4                                                       | 0                             | 0                                                                       |
| 0      | 0                               | 10.4                                                       | 0                             | 0                                                                       |
| 1      | 1                               | 10.4                                                       | 10.4                          | 0.416                                                                   |
| 2      | 2                               | 10.4                                                       | 20.8                          | 0.832                                                                   |
| 3      | 4                               | 10.4                                                       | 41.6                          | 1.664                                                                   |
| 4      | 8                               | 10.4                                                       | 83.2                          | 3.328                                                                   |
| 5      | 4                               | 52.0                                                       | 208                           | 8.320                                                                   |
| 6      | 8                               | 52.0                                                       | 416                           | 16.64                                                                   |
| 7      | 16                              | 52.0                                                       | 832                           | 33.28                                                                   |
| 8      | 32                              | 52.0                                                       | 1664                          | 66.56                                                                   |

### Generation of WR 178,460 Standard Curve Samples

| Sample | Volume Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Mass Spiked<br>( $\mu$ g) | Standard Curve Sample<br>Nominal Concentration<br>( $\mu$ g/ml) |
|--------|-----------------------------|----------------------------------------------------|---------------------------|-----------------------------------------------------------------|
| 00     | 0                           | 10.2                                               | 0                         | 0                                                               |
| 0      | 0                           | 10.2                                               | 0                         | 0                                                               |
| 1      | 1                           | 10.2                                               | 10.2                      | 0.408                                                           |
| 2      | 2                           | 10.2                                               | 20.4                      | 0.816                                                           |
| 3      | 4                           | 10.2                                               | 40.8                      | 1.632                                                           |
| 4      | 8                           | 51.0                                               | 81.6                      | 3.264                                                           |
| 5      | 4                           | 51.0                                               | 204                       | 8.16                                                            |
| 6      | 8                           | 51.0                                               | 408                       | 16.32                                                           |
| 7      | 16                          | 51.0                                               | 816                       | 32.64                                                           |
| 8      | 32                          | 51.0                                               | 1632                      | 65.28                                                           |

### G. QUALITY CONTROL

#### 1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

#### 2. Pipette Calibration

See ASOP 2C-1.1.

#### 3. Balance Calibration

See ASOP 2C-2.1.

### H. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in bile samples. Bile (25  $\mu$ l) and reference (25  $\mu$ l water) samples were spiked with corresponding amounts of halofantrine and WR 178,460 (as free bases) prior to addition of acetonitrile precipitant. Each bile and reference sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added to 150  $\mu$ l of the transferred supernatant (after step 5, not in step 3).

### I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 25  $\mu$ l bile specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

## Generation of Halofantrine Precision Samples

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>( $\mu$ l) | Precision Sample<br>Nominal Concentration<br>( $\mu$ g/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------------|------------------------------------------------------------|
| XL   | 2                              | 11.8                                               | 25                              | 0.944                                                      |
| Low  | 10                             | 11.8                                               | 25                              | 4.72                                                       |
| Med. | 6                              | 59.0                                               | 25                              | 14.16                                                      |
| Hi   | 12                             | 59.0                                               | 25                              | 28.32                                                      |

## Generation of WR 178,460 Precision Samples

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>( $\mu$ l) | Precision Sample<br>Nominal Concentration<br>( $\mu$ g/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------------|------------------------------------------------------------|
| XL   | 2                              | 10.7                                               | 25                              | 0.856                                                      |
| Low  | 10                             | 10.7                                               | 25                              | 4.28                                                       |
| Med. | 6                              | 53.5                                               | 25                              | 12.84                                                      |
| Hi   | 12                             | 53.5                                               | 25                              | 25.68                                                      |

## J. RESULTS

## 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

## 2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 2.

## 3. LOW POINT VALIDATION

Results for this evaluation appears in Table 3.

## 4. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4A-D.

## 5. RECOVERY

Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
HALOFANTRINE (FREE BASE) RAT BILE  
PRECIPITATION ASSAY,  
STUDY REPORT 17, SUPPLEMENT NO. III

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |        | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(µg/ml) |
|---------------------------|--------------------------|--------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(µg/ml) |        |                           |                                        |
| 0                         | 0                        | -      | -                         | -                                      |
| 10.4                      | 0.416                    | 0.080  | 0.080                     | 0.460                                  |
| 20.8                      | 0.832                    | 0.166  | 0.166                     | 0.848                                  |
| 41.6                      | 1.664                    | 0.336  | 0.336                     | 1.62                                   |
| 83.2                      | 3.328                    | 0.652  | 0.652                     | 3.04                                   |
| 208                       | 8.32                     | 1.770  | 1.770                     | 8.10                                   |
| 416                       | 16.64                    | 3.632  | 3.632                     | 16.5                                   |
| 832                       | 33.28                    | 7.509  | 7.509                     | 34.0                                   |
| 1664                      | 66.56                    | 14.691 | 14.691                    | 66.5                                   |

Regression equation:\*\*\*

$$y = 0.221x - 0.0217, r^2 = 0.9995$$

\* Into 25 µl of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight = 1/y.

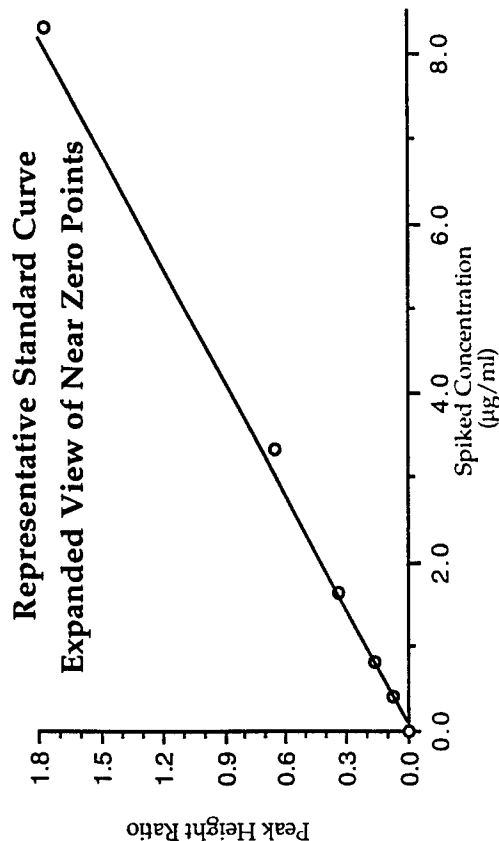
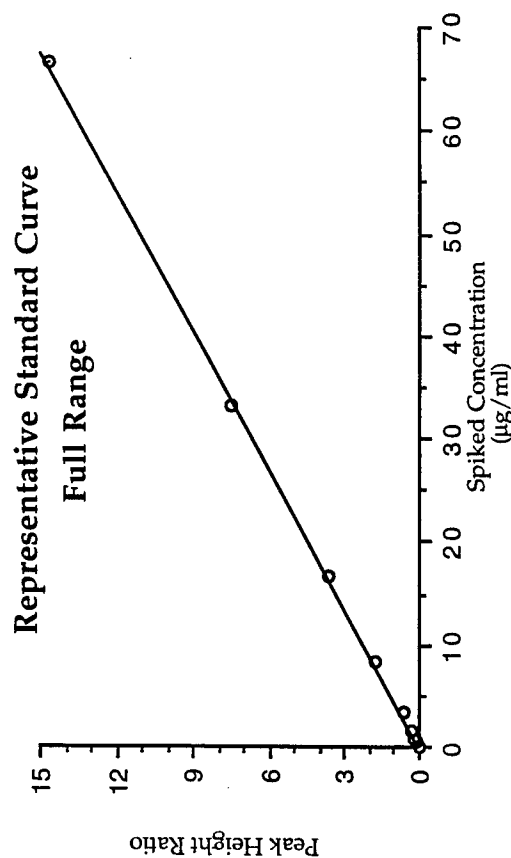


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
WR 178,460 (FREE BASE) RAT BILE PRECIPITATION  
ASSAY, STUDY REPORT 17, SUPPLEMENT NO. III

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |        | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(µg/ml) |
|---------------------------|--------------------------|--------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(µg/ml) |        |                           |                                        |
| 0                         | 0                        | -      | -                         | -                                      |
| 10.2                      | 0.408                    | 0.104  | 0.104                     | 0.466                                  |
| 20.4                      | 0.816                    | 0.201  | 0.201                     | 0.82                                   |
| 40.8                      | 1.632                    | 0.401  | 0.401                     | 1.55                                   |
| 81.6                      | 3.264                    | 0.805  | 0.805                     | 3.02                                   |
| 204                       | 8.16                     | 2.150  | 2.150                     | 7.92                                   |
| 408                       | 16.32                    | 4.416  | 4.416                     | 16.2                                   |
| 816                       | 32.64                    | 9.155  | 9.155                     | 33.4                                   |
| 1632                      | 65.28                    | 17.879 | 17.879                    | 65.2                                   |

Regression equation:\*\*\*

$$y = 0.243x - 0.0192, \quad r^2 = 0.9996$$

\* Into 25 µl of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

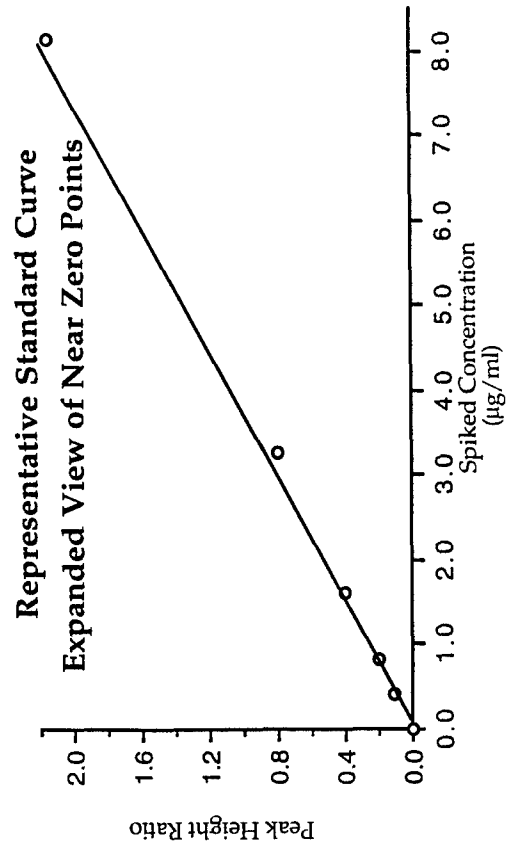
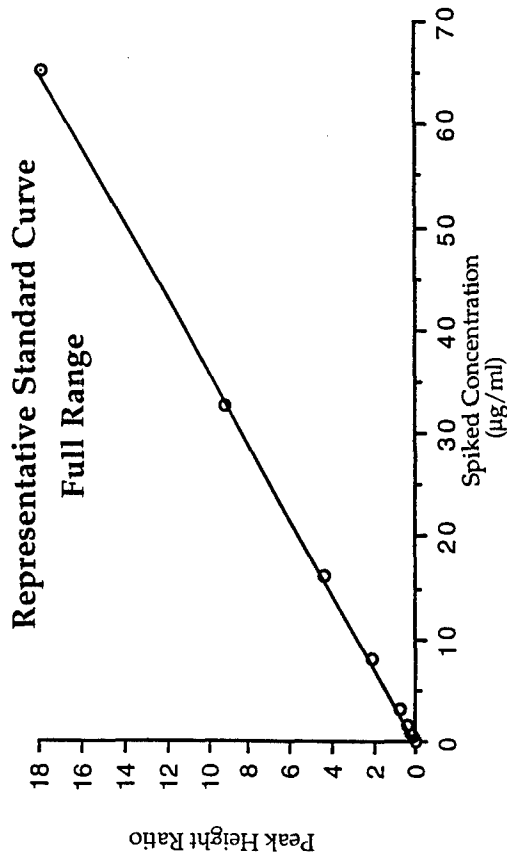


**TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE  
AND WR 178,460 AS FREE BASES RAT PERFUSATE  
EXTRACTION ASSAY, STUDY REPORT 17B, SUPPLEMENT II**

Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|----------------|------------|------------|---------------------------------|
| <u>Halofantrine</u>    |                |            |            |                                 |
| 3/23/94                | 1(interday 1)  | 0.21963467 | -0.0108649 | 0.99934181                      |
| 3/23/96                | 2(intraday)    | 0.22128084 | -0.0216939 | 0.99950683                      |
| 3/24/96                | 3(interday 2)  | 0.2229092  | -0.0150255 | 0.99911694                      |
| 3/26/94                | 4(interday 3)  | 0.22293755 | -0.0162028 | 0.99973786                      |
| <u>WR 178460</u>       |                |            |            |                                 |
| 3/23/94                | 1A(interday 1) | 0.2606906  | -0.0236581 | 0.99780183                      |
| 3/23/96                | 2A(intraday)   | 0.27455641 | -0.0239993 | 0.99946485                      |
| 3/24/96                | 3A(interday 2) | 0.26407302 | -0.0230908 | 0.99855242                      |
| 3/26/94                | 4A(interday 3) | 0.2391856  | -0.0166697 | 0.99554945                      |

Halofantrine Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |       |        |       |       |       |       |       |
|-------------------|---------------------------------------|-------|--------|-------|-------|-------|-------|-------|
|                   | 0.416                                 | 0.832 | 1.664  | 3.328 | 8.32  | 16.64 | 33.28 | 66.56 |
|                   | Back Calculated Concentration (µg/ml) |       |        |       |       |       |       |       |
| 1                 | 0.459                                 | 0.864 | 1.63   | 3.17  | 7.77  | 16.3  | 33.2  | 67.7  |
| 2                 | 0.460                                 | 0.848 | 1.62   | 3.04  | 8.10  | 16.5  | 34.0  | 66.5  |
| 3                 | 0.458                                 | 0.897 | 1.62   | 2.95  | 8.08  | 16.2  | 34.2  | 66.8  |
| 4                 | 0.472                                 | 0.817 | 1.61   | 3.12  | 8.19  | 16.6  | 33.2  | 67.1  |
| Mean              | 0.462                                 | 0.857 | 1.62   | 3.07  | 8.04  | 16.4  | 33.7  | 67.0  |
| S.D.              | 0.0066                                | 0.033 | 0.0082 | 0.096 | 0.183 | 0.183 | 0.526 | 0.512 |
| Percent C.V.      | 1.42                                  | 3.89  | 0.504  | 3.14  | 2.28  | 1.11  | 1.56  | 0.764 |
| Percent R.E.      | 11.1                                  | 2.94  | -2.64  | -7.75 | -3.43 | -1.44 | 1.11  | 0.699 |

WR 178460 Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |       |       |       |       |       |        |       |
|-------------------|---------------------------------------|-------|-------|-------|-------|-------|--------|-------|
|                   | 0.408                                 | 0.816 | 1.632 | 3.264 | 8.16  | 16.32 | 32.64  | 65.28 |
|                   | Back Calculated Concentration (µg/ml) |       |       |       |       |       |        |       |
| 1A                | 0.474                                 | 0.839 | 1.56  | 3.26  | 7.26  | 15.5  | 32.3   | 67.6  |
| 2A                | 0.466                                 | 0.820 | 1.55  | 3.02  | 7.92  | 16.2  | 33.4   | 65.2  |
| 3A                | 0.500                                 | 0.856 | 1.49  | 2.91  | 7.82  | 15.6  | 33.7   | 65.7  |
| 4A                | 0.475                                 | 0.801 | 1.70  | 2.93  | 7.92  | 16.2  | 29.9   | 69.1  |
| Mean              | 0.479                                 | 0.829 | 1.58  | 3.03  | 7.73  | 15.9  | 32.3   | 66.9  |
| S.D.              | 0.015                                 | 0.024 | 0.089 | 0.161 | 0.317 | 0.377 | 1.73   | 1.79  |
| Percent C.V.      | 3.08                                  | 2.87  | 5.64  | 5.30  | 4.10  | 2.38  | 5.34   | 2.68  |
| Percent R.E.      | 17.3                                  | 1.59  | -3.49 | -7.17 | -5.27 | -2.73 | -0.965 | 2.48  |

**TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) RAT BILE PRECIPITATION ASSAY**

| Spiked Concentration | HALOFANTRINE<br>(free base)<br>(0.416 µg/ml) |          | WR 178,460<br>(free base)<br>(0.408 µg/ml) |          |
|----------------------|----------------------------------------------|----------|--------------------------------------------|----------|
|                      | Measured Concentrations<br>(µg/ml)           |          |                                            |          |
|                      | Interday                                     | Intraday | Interday                                   | Intraday |
|                      | 0.459                                        | 0.480    | 0.474                                      | 0.524    |
|                      | 0.458                                        | 0.418    | 0.500                                      | 0.465    |
|                      | 0.472                                        | 0.523    | 0.475                                      | 0.458    |
|                      |                                              | 0.451    |                                            | 0.423    |
|                      |                                              | 0.537    |                                            | 0.462    |
|                      |                                              | 0.451    |                                            | 0.477    |
| Mean                 | 0.463                                        | 0.477    | 0.483                                      | 0.468    |
| Standard Deviation   | 0.008                                        | 0.046    | 0.015                                      | 0.033    |
| Percent C.V.         | 1.69                                         | 9.64     | 3.05                                       | 7.01     |
| Percent R.E.         | 11.3                                         | 14.6     | 18.4                                       | 14.7     |

**TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT BILE  
PRECIPITATION ASSAY**

Interday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 0.944                         | 4.72  | 14.16 | 28.32 |
| Measured Concentrations (µg/ml) |                  |                               |       |       |       |
| 1                               | 1                | 0.910                         | 4.45  | 13.6  | 27.5  |
|                                 | 2                | 1.01                          | 4.46  | 13.9  | 27.1  |
| 3                               | 1                | 0.870                         | 4.32  | 13.1  | 26.9  |
|                                 | 2                | 1.05                          | 4.48  | 13.6  | 28.9  |
| 4                               | 1                | 0.961                         | 4.08  | 13.0  | 27.4  |
|                                 | 2                | 0.988                         | 4.38  | 13.7  | 28.1  |
| Mean                            |                  | 0.965                         | 4.36  | 13.5  | 27.7  |
| S.D.                            |                  | 0.0661                        | 0.15  | 0.354 | 0.737 |
| Percent C.V.                    |                  | 6.85                          | 3.44  | 2.63  | 2.67  |
| Percent R.E.                    |                  | 2.21                          | -7.59 | -4.78 | -2.37 |

**TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT BILE  
PRECIPITATION ASSAY**

Intraday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 0.944                         | 4.72  | 14.16 | 28.32 |
| Measured Concentrations (µg/ml) |                  |                               |       |       |       |
| 2                               | 1                | 0.961                         | 4.38  | 13.4  | 27.0  |
|                                 | 2                | 0.948                         | 4.20  | 13.4  | 28.2  |
|                                 | 3                | 0.966                         | 4.43  | 13.3  | 27.8  |
|                                 | 4                | 1.03                          | 4.49  | 13.6  | 28.8  |
|                                 | 5                | 0.934                         | 4.48  | 13.5  | 28.1  |
|                                 | 6                | 1.00                          | 4.32  | 14.0  | 27.1  |
| Mean                            |                  | 0.973                         | 4.38  | 13.5  | 27.8  |
| S.D.                            |                  | 0.036                         | 0.110 | 0.25  | 0.69  |
| Percent C.V.                    |                  | 3.65                          | 2.51  | 1.85  | 2.48  |
| Percent R.E.                    |                  | 3.09                          | -7.13 | -4.43 | -1.72 |

**TABLE 4C: PRECISION OF WR 178,460 FREE BASE RAT BILE  
PRECIPITATION ASSAY**

Interday Precision WR 178,460 (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 0.860                         | 4.30  | 12.84 | 25.68 |
| Measured Concentrations (µg/ml) |                  |                               |       |       |       |
| 1A                              | 1                | 0.946                         | 4.14  | 12.4  | 25.8  |
|                                 | 2                | 1.08                          | 4.22  | 12.6  | 25.0  |
| 3A                              | 1                | 0.879                         | 3.97  | 12.2  | 25.1  |
|                                 | 2                | 0.947                         | 4.15  | 12.2  | 26.7  |
| 4A                              | 1                | 0.810                         | 3.85  | 12.7  | 26.1  |
|                                 | 2                | 0.797                         | 3.96  | 13.0  | 26.2  |
| Mean                            |                  | 0.910                         | 4.05  | 12.5  | 25.8  |
| S.D.                            |                  | 0.105                         | 0.142 | 0.313 | 0.662 |
| Percent C.V.                    |                  | 11.6                          | 3.52  | 2.50  | 2.56  |
| Percent R.E.                    |                  | 6.29                          | -5.41 | -2.21 | 0.532 |

**TABLE 4D: PRECISION OF WR 178,460 FREE BASE RAT BILE  
PRECIPITATION ASSAY**

Intraday Precision WR 178,460 (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 0.856                         | 4.28  | 12.84 | 25.68 |
| Measured Concentrations (µg/ml) |                  |                               |       |       |       |
| 2A                              | 1                | 0.892                         | 3.99  | 12.4  | 25.5  |
|                                 | 2                | 0.856                         | 3.89  | 12.5  | 26.2  |
|                                 | 3                | 0.849                         | 4.15  | 12.4  | 25.5  |
|                                 | 4                | 0.900                         | 4.09  | 12.4  | 26.5  |
|                                 | 5                | 0.820                         | 4.06  | 12.4  | 25.6  |
|                                 | 6                | 0.914                         | 3.94  | 12.8  | 24.7  |
| Mean                            |                  | 0.872                         | 4.02  | 12.5  | 25.7  |
| S.D.                            |                  | 0.036                         | 0.098 | 0.16  | 0.63  |
| Percent C.V.                    |                  | 4.12                          | 2.43  | 1.28  | 2.45  |
| Percent R.E.                    |                  | 1.85                          | -6.07 | -2.47 | -0.05 |

**TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT BILE BY PRECIPITATION**

| SAMPLE<br>ID               | SPIKED<br>CONCENTRATION |         | PEAK HEIGHT RATIO |              | MEAN<br>PERCENT<br>RECOVERY |
|----------------------------|-------------------------|---------|-------------------|--------------|-----------------------------|
|                            | Range                   | (µg/ml) | REFERENCE         | BILE         |                             |
| <u>Halofantrine</u>        |                         |         |                   |              |                             |
| 1                          | Extra Low               | 0.944   | 0.111             | 0.130        | 108                         |
| 2                          |                         |         | 0.117             | 0.114        |                             |
| 3                          |                         |         | 0.099             | 0.110        |                             |
| Mean (± SD)                |                         |         | 0.109 ±0.009      | 0.118 ±0.011 |                             |
| 1                          | Low                     | 4.72    | 0.534             | 0.575        | 105                         |
| 2                          |                         |         | 0.563             | 0.587        |                             |
| 3                          |                         |         | 0.547             | 0.569        |                             |
| Mean (± SD)                |                         |         | 0.548 ±0.015      | 0.577 ±0.009 |                             |
| 1                          | Medium                  | 14.16   | 1.760             | 1.880        | 103                         |
| 2                          |                         |         | 1.836             | 1.848        |                             |
| 3                          |                         |         | 1.765             | 1.794        |                             |
| Mean (± SD)                |                         |         | 1.787 ±0.043      | 1.841 ±0.043 |                             |
| 1                          | High                    | 28.32   | 3.537             | 3.867        | 97.8                        |
| 2                          |                         |         | 3.951             | 3.773        |                             |
| 3                          |                         |         | 4.032             | 3.629        |                             |
| Mean (± SD)                |                         |         | 3.840 ±0.266      | 3.756 ±0.120 |                             |
| AVERAGE MEAN RECOVERY =    |                         |         |                   |              | 104                         |
| <u>WR 178.460</u>          |                         |         |                   |              |                             |
| 1                          | Extra Low               | 0.856   | 0.126             | 0.126        | 109                         |
| 2                          |                         |         | 0.141             | 0.133        |                             |
| 3                          |                         |         | 0.105             | 0.147        |                             |
| Mean (± SD)                |                         |         | 0.124 ±0.018      | 0.135 ±0.011 |                             |
| 1                          | Low                     | 4.28    | 0.635             | 0.656        | 101                         |
| 2                          |                         |         | 0.683             | 0.679        |                             |
| 3                          |                         |         | 0.616             | 0.625        |                             |
| Mean (± SD)                |                         |         | 0.645 ±0.035      | 0.653 ±0.027 |                             |
| 1                          | Medium                  | 12.84   | 2.077             | 2.167        | 102                         |
| 2                          |                         |         | 2.109             | 2.108        |                             |
| 3                          |                         |         | 1.990             | 2.047        |                             |
| Mean (± SD)                |                         |         | 2.059 ±0.062      | 2.107 ±0.060 |                             |
| 1                          | High                    | 25.68   | 4.108             | 4.461        | 98.4                        |
| 2                          |                         |         | 4.572             | 4.414        |                             |
| 3                          |                         |         | 4.627             | 4.225        |                             |
| Mean (± SD)                |                         |         | 4.436 ±0.285      | 4.367 ±0.125 |                             |
| OVERALL AVERAGE RECOVERY = |                         |         |                   |              | 103                         |

**LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460  
(AS FREE BASES) IN RAT BILE EXTRACTION ASSAY, STUDY REPORT 17,  
SUPPLEMENT IV**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
4. WR 178,460 (hydrochloride), bottle no. BK 21070, (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
5. WR 122,455 (internal standard), bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

## C. ASSAY CONDITIONS

### 1. DETECTOR

#### Settings

Wavelength: excitation - 300 nm, emission - 375 nm

Sensitivity: high

Range: 4

Response: medium

#### Lamp

Ushio xenon, type UXL-155-LCA(S-LC)

### 2. COLUMN

Axxiom Silica, 5  $\mu$ m particle size, 4.6 x 250 mm  
(Richard Scientific, Novato, CA).

### 3. SOLVENT SYSTEM

Combine and mix H<sub>2</sub>O (800 ml) + (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (20 ml of 1 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) + CH<sub>3</sub>OH (3200 ml).

### 4. FLOW RATE

1.0 ml/min.

5. STOCK SOLUTIONS - Solutions were stored in the -20°C freezer and were checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Solution storage bottles were amber or covered with aluminum foil.

#### A. Stock Solutions

##### i. HALOFANTRINE - (Halofantrine Hydrochloride).

Prep date: 3/1/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------------|
| Standard Curve | 5.60                    | 0.942          | 50.6           | methanol | 104                 |
| Precision      | 6.24                    | 0.942          | 50.0           | methanol | 118                 |

\* = Molecular weights of halofantrine free base/halofantrine hydrochloride

## ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride).

Prep date: 3/1/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------|
| Standard Curve | 5.60                    | 0.924          | 50.6           | methanol | 102           |
| Precision      | 6.03                    | 0.924          | 52.0           | methanol | 107           |

\* = Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

## iii. WR 122,455 (Internal Standard).

Prep date: 3/4/94

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|---------------|-------------------------|---------------|----------------|----------|---------------|
| Internal std. | 5.20                    | 1             | 25             | methanol | 208           |

## B. Working Solutions

## A. Halofantrine and WR 178,460 Mixed Working Solutions

1. High Concentration Working Solution: Combine 8.00 ml each of halofantrine and WR 178,460 (as free bases) stock solutions.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 8.00                | 100            | methanol | 8.32          |
| Precision (Halofantrine)      | 118                   | 8.00                | 100            | methanol | 9.44          |
| Standard Curve (WR 178,460)   | 102                   | 8.00                | 100            | methanol | 8.16          |
| Precision (WR 178,460)        | 107                   | 8.00                | 100            | methanol | 8.56          |

2. Low Concentration Working Solution: Combine 1.00 ml each of halofantrine and WR 178,460 (as free bases) stock standard curve solutions and q.s. to 100 ml.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 104                   | 2.00                | 100            | methanol | 2.08          |
| Precision (Halofantrine)      | 118                   | 2.00                | 100            | methanol | 2.36          |

| Solution Type                  | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>(ng/ml) |
|--------------------------------|------------------------------------------|---------------------------|----------------------|----------|------------------|
| Standard Curve<br>(WR 178,460) | 102                                      | 2.00                      | 100                  | methanol | 2.04             |
| Precision<br>(WR 178,460)      | 107                                      | 2.00                      | 100                  | methanol | 2.14             |

B. WR 122,455 - Internal standard.

Prep date: 3/4/94

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g/ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>( $\mu\text{g/ml}$ ) |
|---------------|------------------------------------------|---------------------------|----------------------|----------|-------------------------------|
| Internal std. | 208                                      | 0.5                       | 100                  | methanol | 1.04                          |

7. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.

- Halofantrine - 6.8 min.
- WR 178,460 - 9.6 min.
- WR 122,455 (Internal Standard) - 11 min.

8. BLANK RAT BILE

Supplied by WRAIR.

9. INJECTION VOLUME

50 - 100  $\mu\text{l}$

10. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of  $1/y_i$ .

11. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat bile were based on the interday and intraday low point validation results (Table 3).)

20.8 ng/ml halofantrine (free base).

20.4 ng/ml WR 178,460 (free base).

## 12. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT

Plasma samples and internal standard spiking volumes were measured with a calibrated (ASOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. The drug is spiked with a Hamilton syringe.

## 13. WISP OPERATING TEMPERATURE

Room temperature.

### D. SAMPLE STORAGE

All samples are to be kept frozen at  $-20^{\circ}\text{C}$  before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

### E. SAMPLE PREPARATION

1. Pipet 100  $\mu\text{l}$  rat bile samples into 16 X 125 silanized tubes on ice. Add 100  $\mu\text{l}$  water.
2. Add 20  $\mu\text{l}$  of 1.04  $\mu\text{g}/\text{ml}$  WR 122,455 internal standard solution. Vortex for 30s.
3. Add 50  $\mu\text{l}$  0.1N NaOH. Vortex 30 s.
4. Add 2.0 ml methyl *t*-butyl ether. Vortex 1 min., twice. Centrifuge for 10 min. at 3000 g.
5. Freeze aqueous layer in dry ice/methanol bath and pour organic layer into a 13 x 100 silanized tube.
6. Repeat steps 4 and 5.
7. Evaporate to dryness. Reconstitute in 4:1 methanol/water (v/v) with a final 0.001% HCl concentration.
8. Transfer supernatant to silanized inserts and inject onto column.

### F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 100  $\mu\text{l}$  bile samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

### Generation of Halofantrine Standard Curve Samples

| Sample | Volume Spiked ( $\mu$ l) | Spiking Solution Concentration ( $\mu$ g/ml) | Mass Spiked (ng) | Standard Curve Sample Nominal Concentration (ng/ml) |
|--------|--------------------------|----------------------------------------------|------------------|-----------------------------------------------------|
| 00     | 0                        | 0                                            | 0                | 0                                                   |
| 0      | 0                        | 0                                            | 0                | 0                                                   |
| 2      | 1                        | 2.08                                         | 2.08             | 20.8                                                |
| 3      | 2                        | 2.08                                         | 4.16             | 41.6                                                |
| 4      | 4                        | 2.08                                         | 8.32             | 83.2                                                |
| 5      | 8                        | 2.08                                         | 16.64            | 166.4                                               |
| 6      | 4                        | 8.32                                         | 33.28            | 333                                                 |
| 7      | 8                        | 8.32                                         | 66.56            | 666                                                 |
| 8      | 16                       | 8.32                                         | 133.12           | 1331                                                |

### Generation of WR 178,460 Standard Curve Samples

| Sample | Volume Spiked ( $\mu$ l) | Spiking Solution Concentration ( $\mu$ g/ml) | Mass Spiked (ng) | Standard Curve Sample Nominal Concentration (ng/ml) |
|--------|--------------------------|----------------------------------------------|------------------|-----------------------------------------------------|
| 00     | 0                        | 0                                            | 0                | 0                                                   |
| 0      | 0                        | 0                                            | 0                | 0                                                   |
| 2      | 1                        | 2.04                                         | 2.04             | 20.4                                                |
| 3      | 2                        | 2.04                                         | 4.08             | 40.8                                                |
| 4      | 4                        | 2.04                                         | 8.16             | 81.6                                                |
| 5      | 8                        | 2.04                                         | 16.32            | 163                                                 |
| 6      | 4                        | 8.16                                         | 32.64            | 326                                                 |
| 7      | 8                        | 8.16                                         | 65.28            | 653                                                 |
| 8      | 16                       | 8.16                                         | 130.56           | 1306                                                |

## G. QUALITY CONTROL

### 1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

### 2. Pipette Calibration

See ASOP 2C-1.1.

### 3. Balance Calibration

See ASOP 2C-2.1.

## H. RECOVERY

Assay recovery was assessed at four different halofantrine and WR 178,460 (as free bases) concentrations (equal to precision sample nominal concentrations) by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in bile (100  $\mu$ l) samples. The bile samples were generated by spiking on ice 100  $\mu$ l of blank rat

bile to which water (100  $\mu$ l) has been added with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). Each bile sample was prepared as described in "Sample Preparation" above, except the internal standard was added to the transferred organic layer prior to evaporation. The reference samples (100  $\mu$ l rat bile) were generated by spiking methyl *t*-butyl ether extracts of rat bile (obtained as described in "Sample Preparation" above) with appropriate amounts of drug and metabolite (at nominal concentrations identical to precision sample generation). These reference samples were then prepared by completion of the sample preparation procedure as described (addition of IS, evaporation and reconstitution).

## I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 100  $\mu$ l bile specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

### Generation of Halofantrine Precision Samples

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Control Volume<br>( $\mu$ l) | Precision Sample Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|-------------------------------------------------|------------------------------|---------------------------------------------------|
| XL   | 2                           | 2.36                                            | 100                          | 47.2                                              |
| Low  | 4                           | 2.36                                            | 100                          | 94.4                                              |
| Med. | 4                           | 9.44                                            | 100                          | 378                                               |
| Hi   | 10                          | 9.44                                            | 100                          | 944                                               |

### Generation of WR 178,460 Precision Samples

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Control Volume<br>( $\mu$ l) | Precision Sample Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|-------------------------------------------------|------------------------------|---------------------------------------------------|
| XL   | 2                           | 2.14                                            | 100                          | 42.8                                              |
| Low  | 4                           | 2.14                                            | 100                          | 85.6                                              |
| Med. | 4                           | 8.56                                            | 100                          | 342                                               |
| Hi   | 10                          | 8.56                                            | 100                          | 856                                               |

## J. RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appear in Table 2.

3. LOW POINT VALIDATION

Results for this evaluation appear in Table 3.

3. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4A-D.

4. RECOVERY

Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
HALOFANTRINE (FREE BASE) RAT BILE  
EXTRACTION ASSAY,  
STUDY REPORT 17, SUPPLEMENT NO. 4

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) |       |                           |                                        |
| 0                         | 0                        | 0     | -                         | -                                      |
| 2.08                      | 20.8                     | 0.040 | 22.7                      |                                        |
| 4.16                      | 41.6                     | 0.110 | 46.8                      |                                        |
| 8.32                      | 83.2                     | 0.193 | 75.3                      |                                        |
| 16.64                     | 166                      | 0.453 | 165                       |                                        |
| 33.28                     | 333                      | 0.774 | 275                       |                                        |
| 66.56                     | 666                      | 1.957 | 682                       |                                        |
| 133.12                    | 1331                     | 4.025 | 1390                      |                                        |

Regression equation:\*\*\*

$$y = 0.00291x - 0.00261, \quad r^2 = 0.9920$$

\* Into 100  $\mu$ l of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

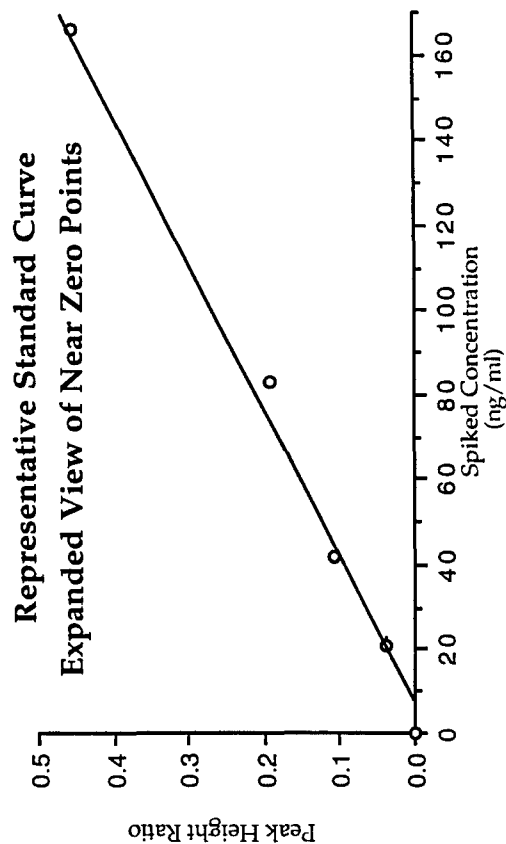
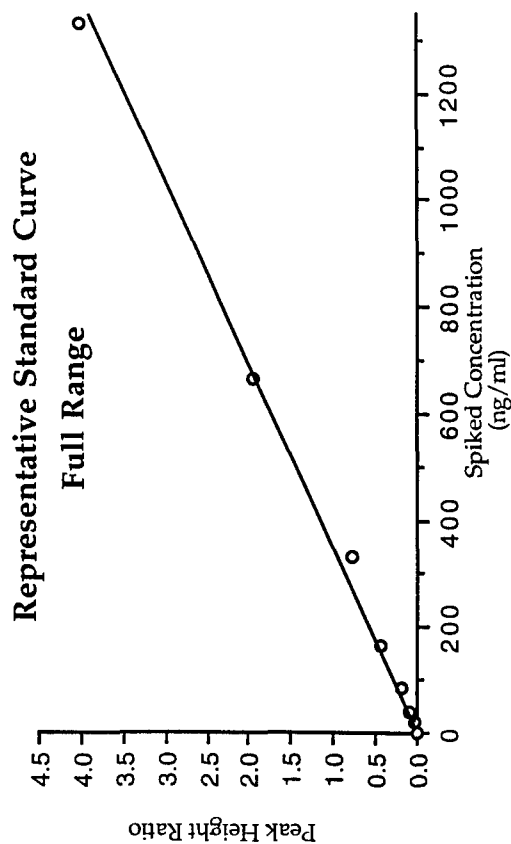


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
WR 178,460 (FREE BASE) RAT BILE EXTRACTION  
ASSAY,  
STUDY REPORT 17, SUPPLEMENT NO. 4

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) |       |                           |                                        |
| 0                         | 0                        | 0     | -                         |                                        |
| 2.04                      | 20.4                     | 0.124 | 21.3                      |                                        |
| 4.08                      | 40.8                     | 0.216 | 40.9                      |                                        |
| 8.16                      | 81.6                     | 0.400 | 80.2                      |                                        |
| 16.32                     | 163                      | 0.813 | 169                       |                                        |
| 32.64                     | 326                      | 1.481 | 311                       |                                        |
| 65.28                     | 653                      | 3.004 | 637                       |                                        |
| 130.56                    | 1306                     | 6.264 | 1330                      |                                        |

Regression equation:  
 $y = 0.00468x + 0.0245$ ,  $r^2 = 0.9991$

\* Into 100  $\mu$ l of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

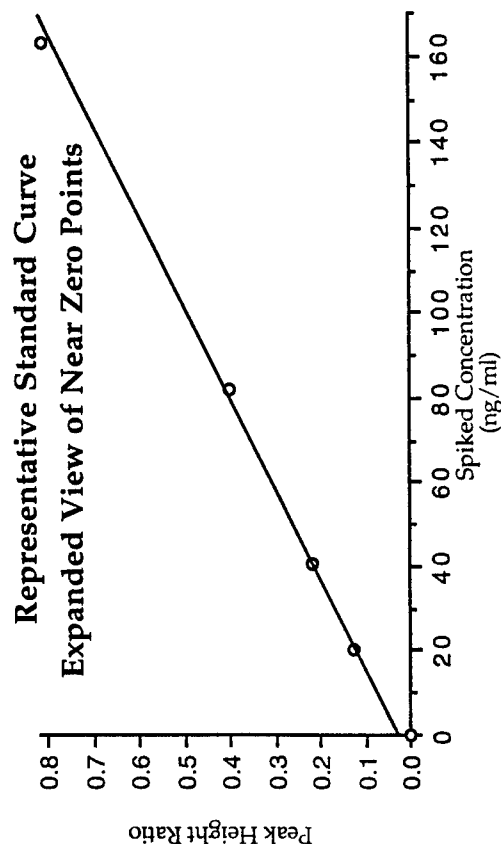
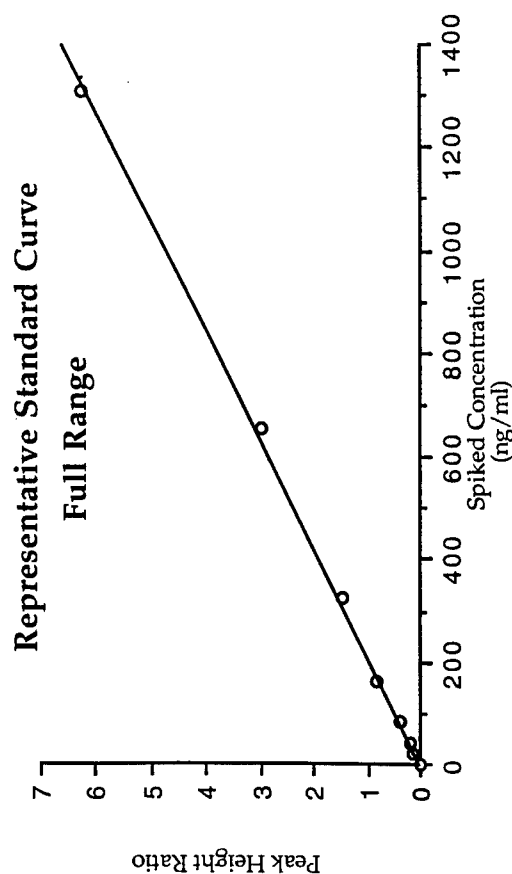


**TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE  
AND WR 178,460 AS FREE BASES RAT BILE EXTRACTION  
ASSAY, STUDY REPORT 17B, SUPPLEMENT IV**

Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|----------------|------------|------------|---------------------------------|
| <u>Halofantrine</u>    |                |            |            |                                 |
| 7/15/94                | 1(Intraday)    | 0.0038786  | -0.0225222 | 0.9990543                       |
| 7/19/94                | 2(Interday 1)  | 0.00334147 | -0.0065534 | 0.99197203                      |
| 7/21/94                | 3(Interday 2)  | 0.00389226 | -0.0694891 | 0.99052267                      |
| 8/5/94                 | 4(Interday 3)  | 0.00290851 | -0.0260724 | 0.992005                        |
| <u>WR 178460</u>       |                |            |            |                                 |
| 7/15/94                | 1A(Intraday)   | 0.00487798 | -0.0318817 | 0.99871791                      |
| 7/19/94                | 2A(Interday 1) | 0.00490082 | -0.0106928 | 0.99079816                      |
| 7/21/94                | 3A(Interday 2) | 0.00500511 | -0.0424384 | 0.99768128                      |
| 8/5/94                 | 4A(Interday 3) | 0.00467863 | 0.02454114 | 0.99907353                      |

Halofantrine Back Calculated Standard Calibrators

| Validation<br>Run                     | Spiked Concentration (ng/ml) |      |       |       |       |       |      |
|---------------------------------------|------------------------------|------|-------|-------|-------|-------|------|
|                                       | 20.8                         | 41.6 | 83.2  | 166   | 333   | 666   | 1331 |
| Back Calculated Concentration (ng/ml) |                              |      |       |       |       |       |      |
| 1                                     | 22.9                         | 43.6 | 80.4  | 139   | 358   | 674   | BC   |
| 2                                     | BC                           | 51.3 | 81.6  | 139   | 324   | 622   | 1420 |
| 3                                     | 22.7                         | 46.8 | 75.3  | 165   | 275   | 682   | 1390 |
| 4                                     | 22.8                         | 40.9 | 77.7  | 169   | 320   | 655   | 1360 |
| Mean                                  | 22.8                         | 45.7 | 78.8  | 153   | 319   | 658   | 1390 |
| S.D.                                  | 0.100                        | 4.47 | 2.82  | 16.2  | 34.1  | 26.7  | 30.0 |
| Percent C.V.                          | 0.439                        | 9.80 | 3.58  | 10.6  | 10.7  | 4.05  | 2.16 |
| Percent R.E.                          | 9.62                         | 9.74 | -5.35 | -7.83 | -4.13 | -1.16 | 4.43 |

WR 178460 Back Calculated Standard Calibrators

| Validation<br>Run                     | Spiked Concentration (ng/ml) |      |       |       |       |       |      |
|---------------------------------------|------------------------------|------|-------|-------|-------|-------|------|
|                                       | 20.4                         | 40.8 | 81.6  | 163   | 326   | 653   | 1306 |
| Back Calculated Concentration (ng/ml) |                              |      |       |       |       |       |      |
| 1A                                    | 23.8                         | 47.1 | 71.8  | 152   | 308   | 592   | 1410 |
| 2A                                    | bc                           | 45.2 | 79.6  | 157   | 311   | 627   | 1350 |
| 3A                                    | 21.3                         | 40.9 | 80.2  | 169   | 311   | 637   | 1330 |
| 4A                                    | 24                           | 35.9 | 80.7  | 165   | 310   | 651   | 1330 |
| Mean                                  | 23.0                         | 42.3 | 78.1  | 161   | 310   | 627   | 1355 |
| S.D.                                  | 1.50                         | 4.98 | 4.21  | 7.68  | 1.41  | 25.2  | 37.9 |
| Percent C.V.                          | 6.53                         | 11.8 | 5.39  | 4.77  | 0.46  | 4.02  | 2.79 |
| Percent R.E.                          | 12.9                         | 3.62 | -4.32 | -1.38 | -4.91 | -4.02 | 3.75 |

**TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) RAT BILE  
EXTRACTION ASSAY**

| Spiked Concentration | HALOFANTRINE<br>(free base)<br>(20.8 ng/ml) |          | WR 178,460<br>(free base)<br>(20.4 ng/ml) |          |
|----------------------|---------------------------------------------|----------|-------------------------------------------|----------|
|                      | Measured Concentrations<br>(ng/ml)          |          |                                           |          |
|                      | Interday                                    | Intraday | Interday                                  | Intraday |
|                      | 22.9                                        | bc       | 23.8                                      | bc       |
|                      | bc                                          | 23.1     | bc                                        | 20.4     |
|                      | 22.7                                        | 22.0     | 21.3                                      | 21.5     |
|                      |                                             | 24.1     |                                           | 19.1     |
|                      |                                             | 22.0     |                                           | 21.3     |
|                      |                                             | 23.7     |                                           | 20.4     |
| Mean                 | 22.8                                        | 23.0     | 22.6                                      | 20.5     |
| Standard Deviation   | 0.141                                       | 0.963    | 1.77                                      | 0.950    |
| Percent CV           | 0.62                                        | 4.19     | 7.84                                      | 4.63     |
| Percent Error        | 9.62                                        | 10.5     | 10.5                                      | 0.69     |

**TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT BILE  
EXTRACTION ASSAY**

Interday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (ng/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 47.2                          | 94.4  | 378.0 | 944   |
| Measured Concentrations (ng/ml) |                  |                               |       |       |       |
| 2                               | 1                | 59.7                          | 86.4  | 382   | bc    |
|                                 | 2                | 48.0                          | 83.4  | 380   | bc    |
| 3                               | 1                | 49.2                          | 90    | 344   | 833   |
|                                 | 2                | 50.7                          | 88.5  | 334   | 850   |
| 4                               | 1                | 40.3                          | 98.7  | 349   | 834   |
|                                 | 2                | 39.6                          | 98    | 336   | 898   |
| Mean                            |                  | 47.9                          | 90.8  | 354   | 854   |
| S.D.                            |                  | 7.42                          | 6.23  | 21.5  | 30.5  |
| Percent C.V.                    |                  | 15.5                          | 6.86  | 6.07  | 3.57  |
| Percent R.E.                    |                  | 1.52                          | -3.78 | -6.31 | -9.56 |

**TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT BILE  
EXTRACTION ASSAY**

Intraday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (ng/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 47.2                          | 94.4  | 378   | 944   |
| Measured Concentrations (ng/ml) |                  |                               |       |       |       |
| 1                               | 1                | 53.0                          | 79.8  | 355   | 1000  |
|                                 | 2                | 36.2                          | 77.7  | 361   | 925   |
|                                 | 3                | 43.2                          | 88.8  | 363   | 917   |
|                                 | 4                | 48.6                          | 87.5  | 337   | 911   |
|                                 | 5                | 46.3                          | 84.4  | 371   | 928   |
|                                 | 6                | 40.6                          | 89.6  | 382   | 901   |
| Mean                            |                  | 44.7                          | 84.6  | 362   | 930   |
| S.D.                            |                  | 5.97                          | 4.93  | 15.2  | 35.5  |
| Percent C.V.                    |                  | 13.4                          | 5.83  | 4.21  | 3.82  |
| Percent R.E.                    |                  | -5.40                         | -10.3 | -4.37 | -1.45 |

**TABLE 4C: INTERDAY PRECISION OF WR 178,460 FREE BASE RAT BILE EXTRACTION ASSAY**

Interday Precision WR 178,460 (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (ng/ml) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 42.8                          | 85.6  | 342   | 856   |
| Measured Concentrations (ng/ml) |                  |                               |       |       |       |
| 2A                              | 1                | 44.4                          | 79.9  | 317   | 806   |
|                                 | 2                | 40.3                          | 81.1  | 336   | 799   |
| 3A                              | 1                | 51.0                          | 93.0  | 332   | 795   |
|                                 | 2                |                               | 89.8  | 351   | 825   |
| 4A                              | 1                | 33.0                          | 83.9  | 320   | 796   |
|                                 | 2                | 36.6                          | 80.7  | 314   | 824   |
| Mean                            |                  | 41.1                          | 84.7  | 328   | 808   |
| S.D.                            |                  | 6.99                          | 5.43  | 14.1  | 13.7  |
| Percent C.V.                    |                  | 17.0                          | 6.41  | 4.28  | 1.70  |
| Percent R.E.                    |                  | -4.07                         | -1.01 | -4.00 | -5.67 |

**TABLE 4D: INTRADAY PRECISION OF WR 178,460 FREE BASE RAT BILE EXTRACTION ASSAY**

Intraday Precision WR 178,460 (Free Base)

| Validation Run                  | QC Sample No. | Spiked Concentrations (ng/ml) |       |       |       |
|---------------------------------|---------------|-------------------------------|-------|-------|-------|
|                                 |               | 42.8                          | 85.6  | 342   | 856   |
| Measured Concentrations (ng/ml) |               |                               |       |       |       |
| 1A                              | 1             | 47.1                          | 63.7  | 333   | 889   |
|                                 | 2             | 37.5                          | 88.3  | 344   | 851   |
|                                 | 3             | 46.3                          | 86.1  | 326   | 836   |
|                                 | 4             | 43.6                          | 88.5  | 334   | 828   |
|                                 | 5             | 49.0                          | 83.6  | 341   | 846   |
|                                 | 6             | 45.5                          | 86.1  | 360   | 832   |
| Mean                            |               | 44.8                          | 82.7  | 340   | 847   |
| S.D.                            |               | 4.01                          | 9.49  | 11.8  | 22.3  |
| Percent C.V.                    |               | 8.94                          | 11.5  | 3.48  | 2.63  |
| Percent R.E.                    |               | 4.75                          | -3.37 | -0.68 | -1.05 |

TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT BILE BY EXTRACTION

| SAMPLE ID                  | SPIKED CONCENTRATION<br>Range (ng/ml) |      | PEAK HEIGHT RATIO |              | MEAN PERCENT RECOVERY |
|----------------------------|---------------------------------------|------|-------------------|--------------|-----------------------|
|                            |                                       |      | REFERENCE         | BILE         |                       |
| <u>Halofantrine</u>        |                                       |      |                   |              |                       |
| 1                          | Extra Low                             | 47.2 | 0.162             | 0.100        | 81.5                  |
| 2                          |                                       |      | 0.159             | 0.144        |                       |
| 3                          |                                       |      | 0.161             | 0.149        |                       |
| Mean (± SD)                |                                       |      | 0.161 ±0.002      | 0.131 ±0.027 |                       |
| 1                          | Low                                   | 94.4 | 0.309             | 0.209        | 68.0                  |
| 2                          |                                       |      | 0.302             | 0.215        |                       |
| 3                          |                                       |      | 0.329             | 0.215        |                       |
| Mean (± SD)                |                                       |      | 0.313 ±0.014      | 0.213 ±0.003 |                       |
| 1                          | Medium                                | 378  | 1.389             | 0.975        | 64.6                  |
| 2                          |                                       |      | 1.429             | 0.89         |                       |
| 3                          |                                       |      | 1.474             | 0.908        |                       |
| Mean (± SD)                |                                       |      | 1.431 ±0.043      | 0.924 ±0.045 |                       |
| 1                          | High                                  | 944  | 3.357             | 2.274        | 67.8                  |
| 2                          |                                       |      | 3.676             | 2.455        |                       |
| 3                          |                                       |      | 3.58              | 2.463        |                       |
| Mean (± SD)                |                                       |      | 3.538 ±0.164      | 2.397 ±0.107 |                       |
| OVERALL AVERAGE RECOVERY = |                                       |      |                   |              | 70.5                  |
| <u>WR 178,460</u>          |                                       |      |                   |              |                       |
| 1                          | Extra Low                             | 42.8 | 0.221             | 0.224        | 90.1                  |
| 2                          |                                       |      | 0.214             | 0.191        |                       |
| 3                          |                                       |      | 0.241             | 0.194        |                       |
| Mean (± SD)                |                                       |      | 0.225 ±0.014      | 0.203 ±0.018 |                       |
| 1                          | Low                                   | 85.6 | 0.391             | 0.381        | 90.0                  |
| 2                          |                                       |      | 0.356             | 0.33         |                       |
| 3                          |                                       |      | 0.412             | 0.332        |                       |
| Mean (± SD)                |                                       |      | 0.386 ±0.028      | 0.348 ±0.029 |                       |
| 1                          | Medium                                | 342  | 1.682             | 1.577        | 90.6                  |
| 2                          |                                       |      | 1.718             | 1.558        |                       |
| 3                          |                                       |      | 1.734             | 1.515        |                       |
| Mean (± SD)                |                                       |      | 1.711 ±0.027      | 1.550 ±0.032 |                       |
| 1                          | High                                  | 856  | 4.051             | 3.771        | 89.7                  |
| 2                          |                                       |      | 4.382             | 3.89         |                       |
| 3                          |                                       |      | 4.298             | 3.757        |                       |
| Mean (± SD)                |                                       |      | 4.244 ±0.172      | 3.806 ±0.073 |                       |
| OVERALL AVERAGE RECOVERY = |                                       |      |                   |              | 90.1                  |

**LABORATORY METHODOLOGY FOR HALOFANTRINE AND WR 178,460  
AS FREE BASES IN RAT LIVER HOMOGENATE ASSAY, STUDY REPORT  
17B, SUPPLEMENT V**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Shimadzu LC-600 Pump (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
3. Shimadzu RF 535 Fluorescence Detector (ISI Instruspec Inc., Walnut Creek, CA) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Halofantrine hydrochloride, bottle no. BB 43807 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
4. WR 178,460 (hydrochloride), bottle no. BK 21070, (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
5. WR 122,455 (internal standard), bottle no. AX 26839 (Walter Reed Army Institute of Research, Washington D.C.), expiration date not available.
6. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).
9. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

### C. ASSAY CONDITIONS

#### 1. DETECTOR

##### Settings

Wavelength: excitation - 300 nm, emission - 375 nm

Sensitivity: high

Range: 4

Response: medium

Lamp: Ushio xenon, type UXL-155-LCA(S-LC)

#### 2. COLUMN

Axxiom Silica, 5  $\mu$ m, 4.6 x 250 mm (Richard Scientific, Novato, CA).

#### 3. SOLVENT SYSTEM

Combine and mix H<sub>2</sub>O (800 ml) + (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (20 ml of 1 M (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) + CH<sub>3</sub>OH (3200 ml).

#### 4. FLOW RATE

1.0 ml/min.

5. SOLUTIONS - Solutions were stored in the -20°C freezer and checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (solutions are discarded when a more than 10% change in the ratio is observed or 6 months after the preparation date). Storage bottles were amber or covered with aluminum foil.

#### A. Stock Solutions

##### i. HALOFANTRINE - (Halofantrine Hydrochloride).

Prep date: 5/9/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------------|
| Standard Curve | 14.3                    | 0.942          | 25.0           | methanol | 539                 |
| Precision      | 14.2                    | 0.942          | 25.0           | methanol | 535                 |

\*= Molecular weights of halofantrine free base/halofantrine hydrochloride

##### ii. HALOFANTRINE METABOLITE- (WR 178,460 Hydrochloride).

Prep date: 5/9/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|----------|---------------------|
| Standard Curve | 14.6                    | 0.924          | 25.0           | methanol | 540                 |
| Precision      | 14.5                    | 0.924          | 25.0           | methanol | 536                 |

\*= Molecular weights of WR 178,460 free base/WR 178,460 hydrochloride

## iii. WR 122,455 (Internal Standard).

Prep date: 3/4/94

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|---------------|-------------------------|---------------|----------------|----------|---------------|
| Internal std. | 5.20                    | 1             | 25             | methanol | 208           |

## B. Working Solutions

## i. Halofantrine and WR 178,460 Mixed Working Solutions

- a. LOW CONCENTRATION WORKING SOLUTION -  
Combine halofantrine and WR 178,460 (as free bases) stock standard curve solutions.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (µg/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 540                   | 5.00                | 25.0           | methanol | 108           |
| Standard Curve (WR 178,460)   | 539                   | 5.00                | 25.0           | methanol | 108           |
| Precision (Halofantrine)      | 535                   | 5.00                | 25.0           | methanol | 107           |
| Precision (WR 178,460)        | 536                   | 5.00                | 25.0           | methanol | 107           |

- b. HIGH CONCENTRATION WORKING SOLUTION - Combine halofantrine and WR 178,460 (as free bases) stock solutions.

| Solution Type                 | Conc. Diluted (µg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent  | Conc. (ng/ml) |
|-------------------------------|-----------------------|---------------------|----------------|----------|---------------|
| Standard Curve (Halofantrine) | 108                   | 8.00                | 20.0           | methanol | 216           |
| Standard Curve (WR 178,460)   | 108                   | 8.00                | 20.0           | methanol | 216           |
| Precision (Halofantrine)      | 107                   | 8.00                | 20.0           | methanol | 214           |
| Precision (WR 178,460)        | 107                   | 8.00                | 20.0           | methanol | 214           |

## ii. WR 122,455 - Internal standard.

| Solution Type | Conc. Diluted (µg/ml) | Volume Diluted (part) | QS Volume (part) | Solvent  | Conc. (µg/ml) |
|---------------|-----------------------|-----------------------|------------------|----------|---------------|
| Internal std. | 208                   | 0.5                   | 10.5             | methanol | 9.90          |

6. RETENTION TIMES (subject to change depending on temperature and column performance). HPLC system is at room temperature.
  - a. Halofantrine - 7.1 min.
  - b. WR 178,460 - 10.8 min.
  - c. WR 122,455 (Internal Standard) - 12.8 min.
7. BLANK RAT LIVER HOMOGENATE  
Supplied by WRAIR.
8. INJECTION VOLUME  
5  $\mu$ l
9. QUANTITATION  
By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by weighted linear regression with a weight of  $1/y_i$ .
10. MINIMUM QUANTITATION LIMIT OF METHOD (The minimum halofantrine and WR 178,460 (as free bases) quantitation limits for the assay of rat liver homogenate were based on the interday and intraday low point validation results (Table 3).)  
0.540  $\mu$ g/ml halofantrine (free base).  
0.540  $\mu$ g/ml WR 178,460 (free base).
11. SAMPLE AND SPIKED SOLUTION VOLUME MEASUREMENT  
Liver homogenate samples and internal standard spiking volumes were measured with a calibrated (SOP 2C-1.1) Rainen, Eppendorf, Gilson Pipetteman or Costar pipetter. Drugs are spiked with Hamilton syringes.
12. WISP OPERATING TEMPERATURE  
Room temperature.
13. LIVER HOMOGENIZATION  
Rat liver (1 g) is combined with 5 ml of buffer (combine 245 ml H<sub>2</sub>O, 5 ml HCl and 250 ml methanol). Samples are homogenized in a Waring Commercial (Waring Products, New Hartford, CN) blender in a Mini Container.

#### D. SAMPLE STORAGE

All samples are to be kept frozen at -70°C before analysis and thawed at room temperature for preparation (within 30 min.) and analysis.

### E. SAMPLE PREPARATION

1. Pipet 0.200 ml rat liver homogenate samples into 13 X 100 silanized tubes.
2. Add 0.6 ml CH<sub>3</sub>CN. Vortex 1 min.
3. Add 40 µl of 10.0 µg/ml WR 122,455 internal standard solution. Vortex for 1 min.
4. Centrifuge at 3000 g for 10 min.
5. Transfer supernatant to silanized inserts and inject 5 µl onto column.

### F. GENERATION OF STANDARD CURVE CALIBRATORS

Spike 0.2 ml rat liver homogenate standard curve samples with halofantrine and WR 178,460 (as free bases) mixed solutions to make a standard curve. This procedure is equivalent to addition of the masses of halofantrine and WR 178,460 (as free bases) shown below. Since 0.200 ml liver homogenate samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 20s.

#### Generation of Halofantrine and WR 178,460 Standard Curve Samples

| Sample | Volume Spiked (µl) | Spiking Solution Concentration (µg/ml) | Mass Spiked (ng) | Standard Curve Sample Nominal Concentration (µg/ml) |
|--------|--------------------|----------------------------------------|------------------|-----------------------------------------------------|
| 00     | 0                  |                                        | 0                | 0                                                   |
| 0      | 0                  |                                        | 0                | 0                                                   |
| 1      | 1.00               | 108                                    | 108              | 0.540                                               |
| 2      | 2.00               | 108                                    | 216              | 1.08                                                |
| 3      | 4.00               | 108                                    | 432              | 2.16                                                |
| 4      | 8.00               | 108                                    | 864              | 4.32                                                |
| 5      | 16.0               | 108                                    | 1728             | 8.64                                                |
| 6      | 16.0               | 216                                    | 3456             | 17.3                                                |
| 7      | 32.0               | 216                                    | 6912             | 34.6                                                |
| 8      | 64.0               | 216                                    | 13824            | 69.1                                                |

### G. QUALITY CONTROL

1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

## 2. Pipette Calibration

See SOP 2C-1.1.

## 3. Balance Calibration

See SOP 2C-2.1.

# H. RECOVERY

Assay recovery was assessed at four different concentrations by comparing the halofantrine and WR 178,460 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in liver homogenate samples. Liver homogenate (200  $\mu$ l) and reference (200  $\mu$ l water) samples were spiked with corresponding amounts of halofantrine and WR 178,460 (as free bases) prior to addition of acetonitrile precipitant. Each liver homogenate and reference sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added to 500  $\mu$ l of the transferred supernatant (after step 5, not in step 3).

# I. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 200  $\mu$ l liver homogenate specimens with halofantrine and WR 178,460 (as free bases) mixed working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

## Generation of Halofantrine and WR 178,460 Precision Samples

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>( $\mu$ l) | Precision Sample<br>Nominal Concentration<br>( $\mu$ g/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------------|------------------------------------------------------------|
| Hi   | 12                             | 214                                                | 200                             | 25.0                                                       |
| Med. | 6                              | 107                                                | 200                             | 5.35                                                       |
| Low  | 10                             | 107                                                | 200                             | 1.07                                                       |

# J. RESULTS

## 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for halofantrine and WR 178,460 (as free bases) appear in Figure 2. Peak height ratios for these calibrators appear in Table 1.

2. PRECISION STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 2.

3. LOW POINT VALIDATION

Results for this evaluation appears in Table 3.

4. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4A-D.

5. RECOVERY

Results for this evaluation appear in Table 5.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
HALOFANTRINE (FREE BASE) RAT LIVER  
HOMOGENATE ASSAY,  
STUDY REPORT 17, SUPPLEMENT NO. V

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE                            |        | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>( $\mu\text{g}/\text{ml}$ ) |
|---------------------------|----------------------------------------------|--------|---------------------------|------------------------------------------------------------|
|                           | CONCENTRATION<br>( $\mu\text{g}/\text{ml}$ ) |        |                           |                                                            |
| 0                         | 0                                            | -      | -                         | -                                                          |
| 108                       | 0.54                                         | 0.169  | 0.169                     | 0.506                                                      |
| 216                       | 1.08                                         | 0.400  | 0.400                     | 1.15                                                       |
| 432                       | 2.16                                         | 0.748  | 0.748                     | 2.12                                                       |
| 864                       | 4.32                                         | 1.494  | 1.494                     | 4.20                                                       |
| 1728                      | 8.64                                         | 3.326  | 3.326                     | 9.31                                                       |
| 3456                      | 17.3                                         | 6.121  | 6.121                     | 17.1                                                       |
| 6912                      | 34.6                                         | 12.324 | 12.324                    | 34.4                                                       |
| 13824                     | 69.1                                         | 24.708 | 24.708                    | 69.0                                                       |

Regression equation:  
 $y = 0.358x - 0.0124, r^2 = 0.9995$

\* Into 200  $\mu\text{l}$  of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

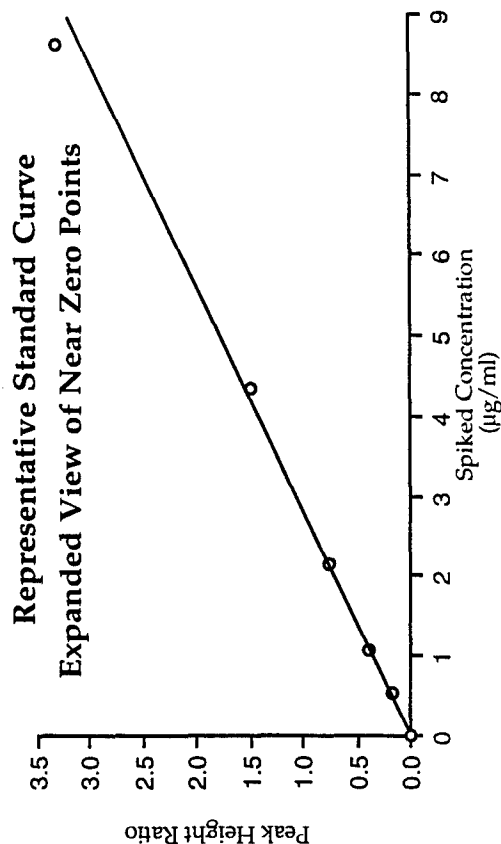
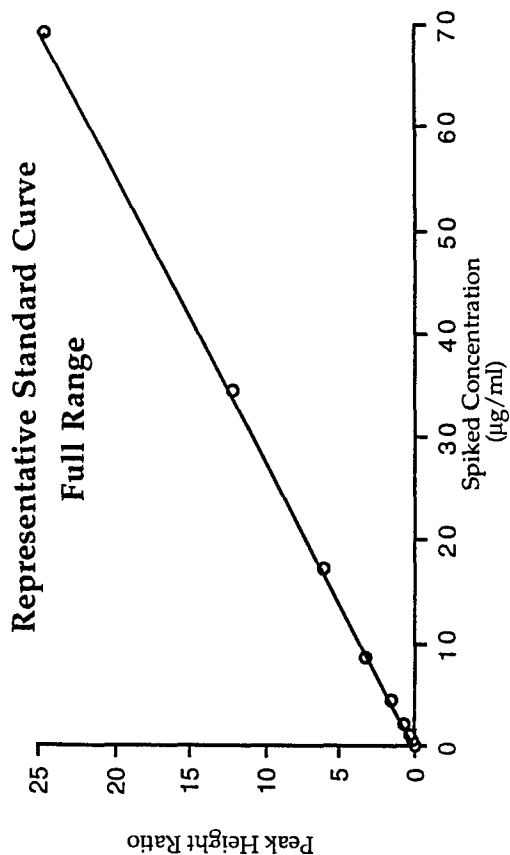


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
WR 178,460 (FREE BASE) RAT LIVER  
HOMOGENATE ASSAY,  
STUDY REPORT 17, SUPPLEMENT NO. V

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |        | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(µg/ml) |
|---------------------------|--------------------------|--------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(µg/ml) |        |                           |                                        |
| 0                         | 0                        | -      | -                         | -                                      |
| 108                       | 0.540                    | 0.297  | 0.561                     |                                        |
| 216                       | 1.08                     | 0.562  | 1.08                      |                                        |
| 432                       | 2.16                     | 1.093  | 2.11                      |                                        |
| 864                       | 4.32                     | 2.135  | 4.14                      |                                        |
| 1728                      | 8.64                     | 4.766  | 9.26                      |                                        |
| 3456                      | 17.3                     | 8.629  | 16.8                      |                                        |
| 6912                      | 34.6                     | 17.635 | 34.3                      |                                        |
| 13824                     | 69.1                     | 35.770 | 69.6                      |                                        |

\*\*\*

Regression equation:  
 $y = 0.514x - 0.0087, r^2 = 0.9994$

\* Into 200 µl of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

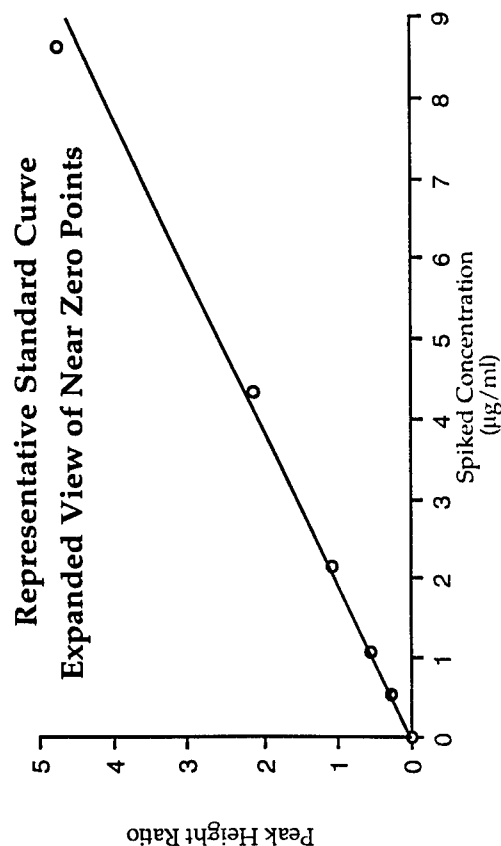
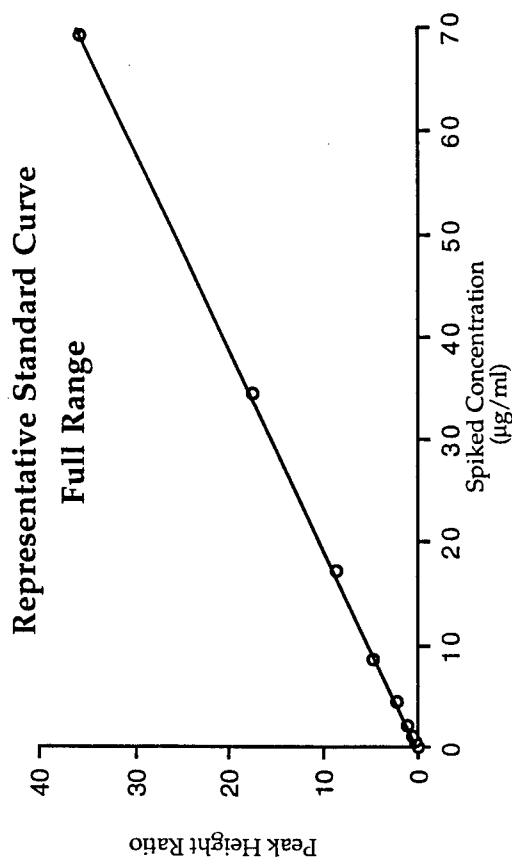


**TABLE 2: PRECISION STANDARD CURVE DATA FOR HALOFANTRINE  
AND WR 178,460 AS FREE BASES RAT LIVER ASSAY, STUDY  
REPORT 17B, SUPPLEMENT V**

Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope       | Intercept   | Coefficient of<br>Determination |
|------------------------|----------------|-------------|-------------|---------------------------------|
| <u>Halofantrine</u>    |                |             |             |                                 |
| 5/12/94                | 1(Intraday)    | 0.358405524 | -0.01242626 | 0.999479418                     |
| 5/19/94                | 2(Interday 1)  | 0.355267367 | 0.012544907 | 0.999903401                     |
| 5/20/96                | 3(Interday 2)  | 0.364534322 | 0.014512231 | 0.99832086                      |
| 5/22/96                | 4(Interday 3)  | 0.353792812 | 0.011080767 | 0.999082954                     |
| <u>WR 178460</u>       |                |             |             |                                 |
| 5/12/94                | 1A(Intraday)   | 0.513864726 | 0.00868     | 0.999386942                     |
| 5/19/94                | 2A(Interday 1) | 0.508007617 | 0.015582886 | 0.999764575                     |
| 5/20/96                | 3A(Interday 2) | 0.521074272 | 0.025879712 | 0.998951121                     |
| 5/22/96                | 4A(Interday 3) | 0.507207281 | 0.000278691 | 0.998773148                     |

Halofantrine Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |       |        |       |       |       |       |        |
|-------------------|---------------------------------------|-------|--------|-------|-------|-------|-------|--------|
|                   | 0.540                                 | 1.08  | 2.16   | 4.32  | 8.64  | 17.3  | 34.6  | 69.1   |
|                   | Back Calculated Concentration (µg/ml) |       |        |       |       |       |       |        |
| 1                 | 0.506                                 | 1.15  | 2.12   | 4.2   | 9.31  | 17.1  | 34.4  | 69.0   |
| 2                 | 0.550                                 | 1.07  | 2.12   | 4.26  | 8.92  | 17.2  | 34.5  | 69.1   |
| 3                 | 0.649                                 | 1.01  | 2.02   | 4.09  | 8.48  | 17.9  | 36.4  | 67.4   |
| 4                 | 0.554                                 | 1.04  | 2.22   | 4.17  | 9.06  | 17.3  | 33.2  | 70.3   |
| Mean              | 0.565                                 | 1.07  | 2.12   | 4.18  | 8.94  | 17.4  | 34.6  | 69.0   |
| S.D.              | 0.0602                                | 0.060 | 0.0816 | 0.071 | 0.348 | 0.359 | 1.32  | 1.19   |
| Percent C.V.      | 10.7                                  | 5.64  | 3.85   | 1.69  | 3.89  | 2.07  | 3.82  | 1.73   |
| Percent R.E.      | 4.58                                  | -1.16 | -1.852 | -3.24 | 3.50  | 0.434 | 0.072 | -0.217 |

WR 178460 Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |        |        |        |       |        |        |       |
|-------------------|---------------------------------------|--------|--------|--------|-------|--------|--------|-------|
|                   | 0.540                                 | 1.08   | 2.16   | 4.32   | 8.64  | 17.3   | 34.6   | 69.1  |
|                   | Back Calculated Concentration (µg/ml) |        |        |        |       |        |        |       |
| 1A                | 0.561                                 | 1.08   | 2.11   | 4.14   | 9.26  | 16.8   | 34.3   | 69.6  |
| 2A                | 0.526                                 | 1.11   | 2.13   | 4.22   | 9.07  | 17.2   | 34.3   | 69.3  |
| 3A                | 0.618                                 | 0.994  | 2.11   | 4.14   | 8.52  | 17.7   | 36.1   | 67.7  |
| 4A                | 0.534                                 | 1.09   | 2.17   | 4.23   | 9.13  | 17.2   | 32.9   | 70.6  |
| Mean              | 0.560                                 | 1.07   | 2.13   | 4.18   | 9.00  | 17.2   | 34.4   | 69.3  |
| S.D.              | 0.0416                                | 0.0512 | 0.0283 | 0.0492 | 0.326 | 0.369  | 1.31   | 1.20  |
| Percent C.V.      | 7.44                                  | 4.79   | 1.33   | 1.18   | 3.63  | 2.14   | 3.81   | 1.74  |
| Percent R.E.      | 3.66                                  | -1.06  | -1.39  | -3.18  | 4.11  | -0.434 | -0.578 | 0.289 |

**TABLE 3: LOW POINT VALIDATION OF HALOFANTRINE AND  
WR 178,460 (AS FREE BASES) RAT LIVER ASSAY**

|                      | HALOFANTRINE<br>(free base)<br>(0.540 µg/ml) |          | WR 178,460<br>(free base)<br>(0.540 µg/ml) |          |
|----------------------|----------------------------------------------|----------|--------------------------------------------|----------|
| Spiked Concentration | Measured Concentrations<br>(µg/ml)           |          |                                            |          |
|                      | Interday                                     | Intraday | Interday                                   | Intraday |
|                      | 0.550                                        | 0.477    | 0.526                                      | 0.530    |
|                      | 0.649                                        | 0.559    | 0.618                                      | 0.570    |
|                      | 0.554                                        | 0.590    | 0.534                                      | 0.639    |
|                      |                                              | 0.514    |                                            | 0.509    |
|                      |                                              | 0.618    |                                            | 0.564    |
|                      |                                              | 0.539    |                                            | 0.568    |
| Mean                 | 0.584                                        | 0.550    | 0.559                                      | 0.563    |
| Standard Deviation   | 0.056                                        | 0.051    | 0.051                                      | 0.044    |
| Percent C.V.         | 9.59                                         | 9.30     | 9.11                                       | 7.88     |
| Percent R.E.         | 8.21                                         | 1.76     | 3.58                                       | 4.32     |

**TABLE 4A: PRECISION OF HALOFANTRINE FREE BASE RAT LIVER ASSAY**

Interday Precision Halofantrine (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/ml) |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|
|                                 |                  | 1.07                          | 5.35  | 24.6  |
| Measured Concentrations (µg/ml) |                  |                               |       |       |
| 2                               | 1                | 1.13                          | 5.36  | 26.0  |
|                                 | 2                | 1.13                          | 5.30  | 26.5  |
| 3                               | 1                | 1.06                          | 5.17  | 25.3  |
|                                 | 2                | 0.983                         | 5.28  | 25.6  |
| 4                               | 1                | 1.04                          | 5.71  | 25.5  |
|                                 | 2                | 1.11                          | 5.34  | 27.0  |
| Mean                            |                  | 1.08                          | 5.36  | 26.0  |
| S.D.                            |                  | 0.0586                        | 0.184 | 0.655 |
| Percent C.V.                    |                  | 5.45                          | 3.43  | 2.52  |
| Percent R.E.                    |                  | 0.514                         | 0.187 | 5.62  |

**TABLE 4B: PRECISION OF HALOFANTRINE FREE BASE RAT LIVER ASSAY**

Intraday Precision Halofantrine (Free Base)

| Validation Run                  | QC Sample No. | Spiked Concentrations (µg/ml) |        |      |
|---------------------------------|---------------|-------------------------------|--------|------|
|                                 |               | 1.07                          | 5.35   | 24.6 |
| Measured Concentrations (µg/ml) |               |                               |        |      |
| 1                               | 1             | 1.21                          | 5.58   | 26.4 |
|                                 | 2             | 1.22                          | 5.57   | 27.1 |
|                                 | 3             | 1.2                           | 5.27   | 26.2 |
|                                 | 4             | 1.23                          | 5.43   | 27.3 |
|                                 | 5             | 1.12                          | 4.98   | 26.5 |
|                                 | 6             | 1.11                          | 5.05   | 25.6 |
| Mean                            |               | 1.18                          | 5.31   | 26.5 |
| S.D.                            |               | 0.053                         | 0.258  | 0.62 |
| Percent C.V.                    |               | 4.46                          | 4.86   | 2.33 |
| Percent R.E.                    |               | 10.4                          | -0.685 | 7.79 |

TABLE 4C: PRECISION OF WR 178,460 FREE BASE RAT LIVER ASSAY

Interday Precision WR 178,460 (Free Base)

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/ml) |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|
|                                 |                  | 1.07                          | 5.35  | 24.6  |
| Measured Concentrations (µg/ml) |                  |                               |       |       |
| 2A                              | 1                | 1.20                          | 5.43  | 23.7  |
|                                 | 2                | 1.21                          | 5.38  | 24.0  |
| 3A                              | 1                | 1.01                          | 5.20  | 23.1  |
|                                 | 2                | 0.992                         | 5.24  | 23.3  |
| 4A                              | 1                | 1.14                          | 5.75  | 23.2  |
|                                 | 2                | 1.17                          | 5.42  | 24.5  |
| Mean                            |                  | 1.12                          | 5.40  | 23.6  |
| S.D.                            |                  | 0.0958                        | 0.195 | 0.543 |
| Percent C.V.                    |                  | 8.55                          | 3.61  | 2.30  |
| Percent R.E.                    |                  | 4.70                          | 0.997 | -3.93 |

TABLE 4D: PRECISION OF WR 178,460 FREE BASE RAT LIVER ASSAY

Intraday Precision WR 178,460 (Free Base)

| Validation Run                  | QC Sample No. | Spiked Concentrations (µg/ml) |       |       |
|---------------------------------|---------------|-------------------------------|-------|-------|
|                                 |               | 1.07                          | 5.35  | 24.6  |
| Measured Concentrations (µg/ml) |               |                               |       |       |
| 1A                              | 1             | 1.12                          | 5.62  | 23.8  |
|                                 | 2             | 1.15                          | 5.56  | 24.6  |
|                                 | 3             | 1.20                          | 5.15  | 23.5  |
|                                 | 4             | 1.16                          | 5.37  | 24.7  |
|                                 | 5             | 1.09                          | 4.96  | 23.7  |
|                                 | 6             | 1.07                          | 5.03  | 23.2  |
| Mean                            |               | 1.13                          | 5.28  | 23.9  |
| S.D.                            |               | 0.048                         | 0.277 | 0.60  |
| Percent C.V.                    |               | 4.23                          | 5.25  | 2.53  |
| Percent R.E.                    |               | 5.76                          | -1.28 | -2.78 |

**TABLE 5: RECOVERY OF HALOFANTRINE AND WR 178,460 AS FREE BASES FROM RAT LIVER HOMOGENATE BY PRECIPITATION**

| SAMPLE ID                  | SPIKED CONCENTRATION |         | PEAK HEIGHT RATIO |                  | MEAN PERCENT RECOVERY |
|----------------------------|----------------------|---------|-------------------|------------------|-----------------------|
|                            | Range                | (µg/ml) | REFERENCE         | LIVER HOMOGENATE |                       |
| Halofantrine               |                      |         |                   |                  |                       |
| 1                          | High                 | 24.6    | 6.698             | 7.384            | 100                   |
| 2                          |                      |         | 7.189             | 6.939            |                       |
| 3                          |                      |         | 6.939             | 6.591            |                       |
| Mean (± SD)                |                      |         | 6.942 ±0.246      | 6.971 ±0.397     |                       |
| 1                          | Medium               | 5.35    | 1.487             | 1.414            | 93.3                  |
| 2                          |                      |         | 1.669             | 1.486            |                       |
| 3                          |                      |         | 1.515             | 1.456            |                       |
| Mean (± SD)                |                      |         | 1.557 ±0.098      | 1.452 ±0.036     |                       |
| 1                          | Low                  | 1.07    | 0.270             | 0.301            | 103.6                 |
| 2                          |                      |         | 0.308             | 0.301            |                       |
| 3                          |                      |         | 0.295             | 0.302            |                       |
| Mean (± SD)                |                      |         | 0.291 ±0.019      | 0.301 ±0.001     |                       |
| OVERALL AVERAGE RECOVERY = |                      |         |                   |                  | 99.1                  |
| <u>WR 178,460</u>          |                      |         |                   |                  |                       |
| 1                          | High                 | 24.6    | 8.725             | 9.330            | 97.8                  |
| 2                          |                      |         | 9.360             | 8.790            |                       |
| 3                          |                      |         | 8.993             | 8.370            |                       |
| Mean (± SD)                |                      |         | 9.026 ±0.319      | 8.830 ±0.481     |                       |
| 1                          | Medium               | 5.35    | 1.944             | 1.885            | 95.0                  |
| 2                          |                      |         | (2.721)bc         | 1.932            |                       |
| 3                          |                      |         | 2.035             | 1.855            |                       |
| Mean (± SD)                |                      |         | 1.990 ±0.064      | 1.891 ±0.039     |                       |
| 1                          | Low                  | 1.07    | 0.403             | 0.362            | 90.9                  |
| 2                          |                      |         | 0.407             | 0.380            |                       |
| 3                          |                      |         | 0.442             | 0.396            |                       |
| Mean (± SD)                |                      |         | 0.417 ±0.021      | 0.379 ±0.017     |                       |
| OVERALL AVERAGE RECOVERY = |                      |         |                   |                  | 94.6                  |

bc = unacceptable chromatogram, data not used in calculations.

**LABORATORY METHODOLOGY FOR WR 6026 AND WR 211,789 (AS FREE BASES) PLASMA ASSAY,\* STUDY REPORT 18**

presented in mid term report

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\* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

**LABORATORY METHODOLOGY FOR STUDY REPORT 19: MEFLOQUINE  
(FREE BASE) HUMAN BLOOD ASSAY**

presented in mid term report

## LABORATORY METHODOLOGY FOR STUDY REPORT 20: ARTELINIC ACID HUMAN PLASMA ASSAY

### A. INSTRUMENTS

1. Waters Associates WISP 710B (Waters Associates, Milford, MA), or equivalent.
2. Shimadzu LC 6A (Shimadzu Scientific Instruments, Inc., Columbia, MD), or equivalent.
3. Kratos Spectroflow 783 UV Absorbance Detector (Kratos Analytical Instruments, Ramsey, NJ), or equivalent.
4. Hewlett-Packard Integrator 3390A (Hewlett-Packard Co., Santa Clara, CA), or equivalent.

### B. REAGENTS

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. Artelinic acid, WR 255663AK (Walter Reed Army Institute of Research, Washington D.C.), bottle number BM04131, expiration date not available.
4. Meclofenamic acid (Internal Standard), (Mylan Pharmaceuticals, and WV), purified by ET Lin.
5. Methanol (Fisher Scientific, Fair Lawn, NJ).
6. Acetonitrile (Fisher Scientific, Fair Lawn, NJ).
7. 85% Phosphoric acid (Fisher Scientific, Fair Lawn, NJ).
8. Type 1 reagent grade water: prepared with a Nanopure II system, Barnstead Co., Boston, MA).

\*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

### C. ASSAY CONDITIONS

#### 1. DETECTOR

Settings

Wavelength: 236 nm

Sensitivity: 0.008 aufs

Lamp: Applied Biosystems deuterium, model 120-LC

#### 2. COLUMN

Axxiom ODS, 5  $\mu$ m particle size, 4.6 x 250 mm (Richard Scientific, Novato, CA).

#### 3. SOLVENT SYSTEM

CH<sub>3</sub>CN/50 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (1:1, v/v), pH adjusted to 5.00 with H<sub>3</sub>PO<sub>4</sub>

#### 4. FLOW RATE

1.0 ml/min

#### 5. STOCK SOLUTIONS - Solutions were stored in a -20°C freezer and checked for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds and internal standard (solutions are discarded when a more than 10% change in the ratio is observed or by 6 months after the preparation date).

##### a. ARTELINIC ACID (Standard Curve Stock Solution)

10.035 mg of artelinic acid dissolved in and q.s. to 50.0 ml with methanol.

Purity factor = 0.9487

Conc. of artelinic acid stock = 0.190 mg/ml

##### b. ARTELINIC ACID (Control Stock Solution)

12.594 mg of artelinic acid dissolved in and q.s. to 50.00 ml with methanol.

Purity factor = 0.9487

Conc. of artelinic acid stock = 0.239 mg/ml

\*Report No. 703 titled "Assay of 10-O-(4'-Carboxybenzyl)dihydroartemisinin Hemihydrate, 4-(10'-Dihydroartemisininoxymethyl)benzoic Acid Hemihydrate (Artelinic Acid), WR-255663AK, BM04131," p. 9.

- c. INTERNAL STANDARD: Meclofenamic acid  
2.945 mg of meclofenamic acid dissolved in 50.0 ml of methanol.  
Conc. of meclofenamic acid stock = 58.9  $\mu\text{g/ml}$
- 6. WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator and discarded when stock solutions were discarded or by 6 months after the preparation date.
  - a. ARTELINIC ACID (Standard Curve Working Solution)  
Dilute 10.0 ml of 0.190 mg/ml artelinic acid stock solution to 24.0 ml with methanol.  
Conc. of artelinic acid working solution = 79.3  $\mu\text{g/ml}$   
  
Dilute 1.0 ml of 79.3  $\mu\text{g/ml}$  artelinic acid working solution to 16 ml with methanol.  
Conc. of artelinic acid working solution = 4.96  $\mu\text{g/ml}$
  - b. ARTELINIC ACID (Control Working Solution)  
Dilute 5.00 ml of 0.239 mg/ml artelinic acid stock solution to 15.0 ml with methanol.  
Conc. of artelinic acid working solution = 79.7  $\mu\text{g/ml}$   
  
Dilute 1.0 ml of 79.7  $\mu\text{g/ml}$  artelinic acid working solution to 16 ml with methanol.  
Conc. of artelinic acid working solution = 4.98  $\mu\text{g/ml}$
  - c. MECLOFENAMIC ACID (Internal standard).  
Dilute 1.0 ml of 58.9  $\mu\text{g/ml}$  meclofenamic acid stock solution to 18 ml with methanol.  
Conc. of meclofenamic acid working solution = 3.27  $\mu\text{g/ml}$
- 7. RETENTION TIMES (subject to change depending on temperature and column performance).
  - a. Artelinic acid - 20 min
  - b. Meclofenamic acid (Internal Standard) - 17 min
- 8. BLANK PLASMA  
  
Human plasma (CPD or CPDA-1 as anticoagulant) was obtained from San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME

60  $\mu$ l

10. QUANTITATION

By peak height ratio of drug relative to internal standard peak.

11. MINIMUM QUANTITATION LIMIT OF METHOD

The minimum quantitation limit, 5.23 ng/ml for artelinic acid, was determined as the artelinic acid concentration at which the signal to noise ratio was at least 3 to 1.

12. SAMPLE VOLUME MEASUREMENT

Plasma sample volumes were measured with a 1000  $\mu$ l Gilson Pipetman. See SOP 3-4 for calibration procedure.

13. WISP OPERATING TEMPERATURE

Room temperature.

14. CARTRIDGE CONDITIONING

QUATERNARY AMINOPROPYL (SAX) ION EXCHANGE CARTRIDGE (500 mg)

- a. Wash cartridge with 3 ml methanol.
- b. Wash with 2 ml 0.1 M HCl.
- c. Wash with 3 ml H<sub>2</sub>O.
- d. Wash with 6 ml 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0.

NH<sub>2</sub> ION EXCHANGE CARTRIDGE (500 mg)

Same as SAX cartridge conditioning, except:

- b. Wash with 6 ml 0.1 M HCl.
- d. Wash with 6 ml 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.01.

D. SAMPLE STORAGE

All samples must be kept frozen at -80°C before analysis and thawed at room temperature for preparation and analysis.

E. SAMPLE PREPARATION

SAX CARTRIDGE ELUTION

1. Pipette 1 ml plasma into culture tube.

2. Spike standard curve samples with 00, 0, 1, 2, or 8  $\mu\text{l}$  of 4.96  $\mu\text{g}/\text{ml}$  artelinic acid working solution or 1, 2, 4, 8, or 16  $\mu\text{l}$  of 79.3  $\mu\text{g}/\text{ml}$  artelinic acid working solution to make a standard curve. This procedure is equivalent to addition of 4.96, 9.92, 19.8, 39.7, 79.3, 159, 317, 635, or 1270 ng of artelinic acid to each tube. Since 1 ml of biological sample is assayed, these amounts correspond to artelinic acid concentrations of 00, 0, 4.96, 9.92, 19.8, 39.7, 79.3, 159, 317, 635, or 1270 ng/ml.
3. Add 50  $\mu\text{l}$  of internal standard solution (3.27  $\mu\text{g}/\text{ml}$  meclofenamic acid). Vortex 10 s.
4. Add 2 ml acetonitrile. Vortex for 1 min. Centrifuge for 10 min at 3000g.
5. Transfer supernatant into clean 12x75 mm tubes.
6. Evaporoate to 200  $\mu\text{l}$  under nitrogen.
7. Add 1 ml water. Vortex for 1 min.
8. Pour sample onto pre-conditioned 500 mg SAX cation-exchange cartridge.
9. Wash cartridge with 3 ml of water followed by 3 ml of acetonitrile by gravity elution.
10. Wash cartridge with 0.5 ml of 0.5 M formic acid in acetonitrile by gravity elution.
11. Elute sample with 2 ml of 0.5 M formic acid in acetonitrile by gravity elution.
12. Evaporate eluent to dryness under nitrogen.
13. Reconstitute sample in 200  $\mu\text{l}$  of 50% acetonitrile, transfer to WISP vial and inject 60  $\mu\text{l}$  onto the HPLC column.

**NH<sub>2</sub> CARTRIDGE ELUTION: SAME AS ABOVE EXCEPT:**

8. Pour sample onto pre-conditioned 500 mg NH<sub>2</sub> cation-exchange cartridge.
9. Wash cartridge with 3 ml of water followed by 3 ml of acetonitrile by gravity elution. Completely dry cartridge on Vacelut.

\*00 = Sample with no drug and no internal standard.

\*\*0 = Sample with no drug but with internal standard.

10. Wash cartridge twice with 0.5 ml of 0.5 M formic acid in acetonitrile by gravity elution. Completely dry cartridge on Vacelut.
11. Elute sample with three 0.5 ml aliquots of 0.5 M formic acid in acetonitrile by gravity elution.
13. Reconstitute sample in 200  $\mu$ l of 50% acetonitrile, transfer to WISP vial and inject 30  $\mu$ l onto the HPLC column.

## F. QUALITY CONTROL

### 1. CONTENT AND FREQUENCY OF BLANKS

No special blank sample was assayed, except for the standard curve and blanks.

### 2. PIPETTE CALIBRATION

See SOP 3-4.1.

### 3. BALANCE CALIBRATION

See SOP 3-19.1

## G. GENERATION OF PRECISION SAMPLES

Precision samples were made and assayed with calibration standards. Samples for precision analysis were prepared by spiking blank 1 ml plasma specimens with artelinic acid control working solution to make final artelinic acid concentrations corresponding to 9.96, 39.8, 398, or 797 ng/ml. See table.

Generation of Precision Samples (NH<sub>2</sub>)

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>(ml) | Precision Sample<br>Nominal Concentration<br>(ng/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------|------------------------------------------------------|
| X-Lo | 1                              | 9.50                                               | 1                         | 9.50                                                 |
| Low  | 4                              | 9.50                                               | 1                         | 38.0                                                 |
| Med. | 4                              | 95.0                                               | 1                         | 380                                                  |
| Hi   | 8                              | 95.0                                               | 1                         | 760                                                  |

### Generation of Precision Samples (SAX)

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>(ml) | Precision Sample<br>Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|----------------------------------------------------|---------------------------|------------------------------------------------------|
| X-Lo | 2                           | 4.98                                               | 1                         | 9.96                                                 |
| Low  | 8                           | 4.98                                               | 1                         | 39.8                                                 |
| Med. | 5                           | 79.7                                               | 1                         | 398                                                  |
| Hi   | 10                          | 79.7                                               | 1                         | 797                                                  |

### H. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at three different concentrations by comparing the artelinic acid to internal standard peak height ratios in solvent to the peak height ratios in plasma. Plasma (1 ml) and solvent (methanol) samples were spiked with corresponding amounts of artelinic acid. Each plasma sample was prepared as described in "Sample Preparation" (Section E), except the internal standard was added after the eluents were collected (step 11) and the solvent samples were not extracted.

### I. GENERATION OF FREEZE THAW SAMPLES

The effect of repeated freeze and thaw cycles on stability of artelinic acid in human plasma samples was determined as follows: Spiked (40 and 800 ng/ml artelinic acid concentrations) pooled biological sample were aliquoted (1 ml) to screw top culture tubes and subjected to five thaw/freeze cycles. Each cycle, a duplicate set of thaw/freeze samples was generated.

Run the study with the following procedure:

- a. Prepare high and low concentration samples labelled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.
- b. Store all samples until frozen at the specified temperature.
- c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 hour.

| Cycle | Keep these<br>samples in freezer | Thaw these<br>samples |
|-------|----------------------------------|-----------------------|
| 1     | 1                                | 2, 3, 4, 5            |
| 2     | 1, 2                             | 3, 4, 5               |
| 3     | 1, 2, 3                          | 4, 5                  |
| 4     | 1, 2, 3, 4                       | 5                     |
| 5     | 1, 2, 3, 4, 5                    | none                  |

- d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

## J. RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for artelinic acid appear in Figure 1. Peak height ratios for these calibrators appear in Table 1.

### 2. RECOVERY

Results for this evaluation appear in Table 2.

### 3. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 3 and 4.

### 4. STANDARD CURVE STUDY STATISTICS

Mean, standard deviation, percent coefficient of variation and percent deviation from corresponding calibrator concentrations of the standard curve calibrators for this study appear in Table 5.

### 5. LOW POINT VALIDATION

Take the 6 back calculated lowest standard calibrator concentrations that were obtained in the interday precision-accuracy study as the quantitation limit interday result. Results appear in Table 6.

### 6. STABILITY

#### a. Freeze and Thaw

Results appear in Table 7.

#### b. Freezer Storage Stability

Freezer storage stability data is presented in Table 8.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR  
ARTELINIC ACID HUMAN PLASMA ASSAY  
STUDY REPORT 20

| SPIKED<br>AMOUNT<br>(ng) | STANDARD<br>CURVE         |                          | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION |
|--------------------------|---------------------------|--------------------------|---------------------------|-----------------------------|
|                          | CONCENTRATION*<br>(ng/ml) | CONCENTRATION<br>(ng/ml) |                           |                             |
| 0                        | 0                         | 0                        | 0                         | -                           |
| 4.96                     | 4.96                      | 4.96                     | 0.031                     | 5.40 <sup>a</sup>           |
| 9.92                     | 9.92                      | 9.92                     | 0.059                     | 10.5 <sup>a</sup>           |
| 19.8                     | 19.8                      | 19.8                     | 0.112                     | 20.2 <sup>a</sup>           |
| 39.7                     | 39.7                      | 39.7                     | 0.209                     | 37.8 <sup>a</sup>           |
| 79.3                     | 79.3                      | 79.3                     | 0.441                     | 80.1 <sup>a</sup>           |
| 159                      | 159                       | 159                      | 0.910                     | 158 <sup>b</sup>            |
| 317                      | 317                       | 317                      | 1.804                     | 315 <sup>b</sup>            |
| 635                      | 635                       | 635                      | 3.779                     | 661 <sup>b</sup>            |
| 1270                     | 1270                      | 1270                     | 7.180                     | 1260 <sup>b</sup>           |

Regression equations:  
\*\*\*

a  $y = 0.00549x + 0.0013$ ,  $r^2 = 0.9989$ ; (Low Range: 0 - 79.3 ng/ml)

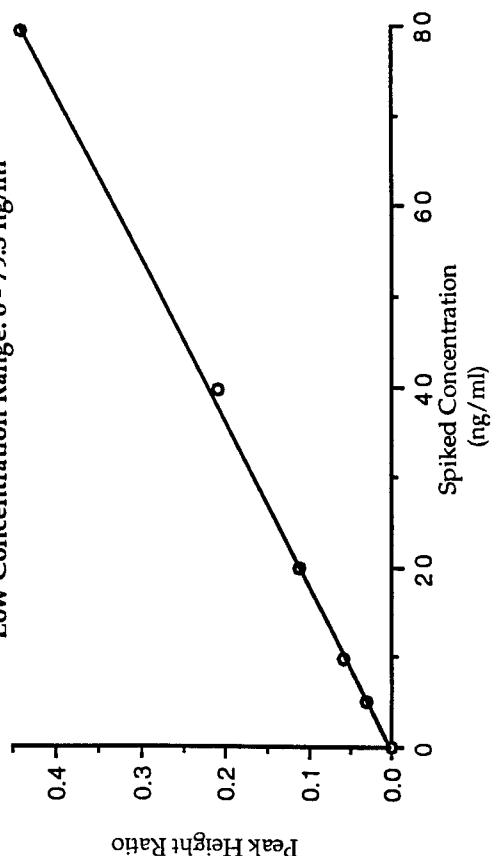
b  $y = 0.00570x + 0.0069$ ,  $r^2 = 0.9994$ ; (High Range: 0 - 1270 ng/ml)

\* When 1 ml of biological sample is used.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 79.3 ng/ml



High Concentration Range: 0 - 1270 ng/ml

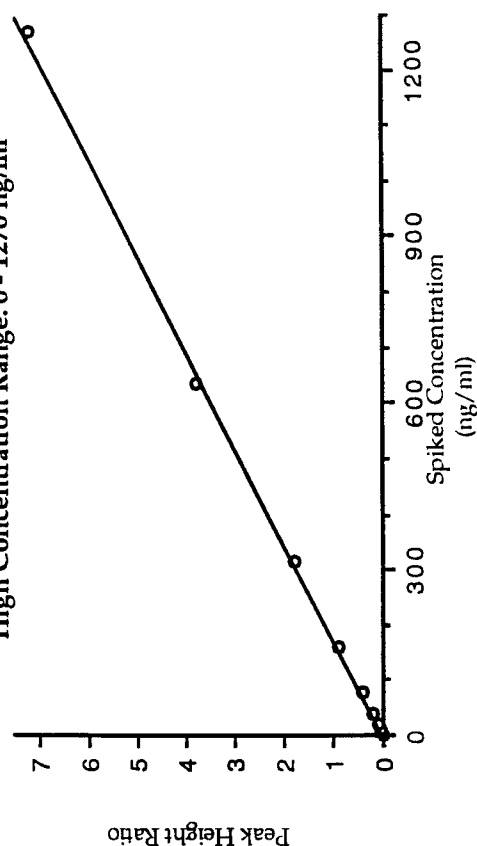


TABLE 2: ARTELINIC ACID MINIMUM QUANTITATION LIMIT

## NH2 Method

| Spiked Concentration | (9.49 ng/ml)                       | (9.5 ng/ml) | (4.75 ng/ml) |
|----------------------|------------------------------------|-------------|--------------|
|                      | Measured Concentrations<br>(ng/ml) |             |              |
|                      | Interday                           | Intraday    | Interday     |
|                      | 9.03                               | 12.1        | 5.20         |
|                      | 9.81                               | 8.35        | 4.55         |
|                      | 9.03                               | 9.72        | 5.20         |
|                      | 9.29                               | 9.03        | bc           |
|                      | 9.77                               | 9.72        | 6.38         |
|                      | 9.82                               | 11.8        | 4.19         |
| Mean                 | 9.46                               | 10.1        | 5.10         |
| Standard Deviation   | 0.386                              | 1.51        | 0.835        |
| Percent CV           | 4.09                               | 14.9        | 16.4         |
| Percent Error        | -0.334                             | 6.53        | 7.45         |

## SAX Method

| Spiked Concentration | (9.92 ng/ml)                       | (9.96 ng/ml) | (4.96 ng/ml) |
|----------------------|------------------------------------|--------------|--------------|
|                      | Measured Concentrations<br>(ng/ml) |              |              |
|                      | Interday                           | Intraday     | Interday     |
|                      | 9.97                               | 9.95         | 6.13         |
|                      | bc                                 | 10.5         | 6.30         |
|                      | 8.75                               | 9.77         | 5.09         |
|                      | 8.92                               | 8.32         | bc           |
|                      | 9.98                               | 10.1         | bc           |
|                      | 9.51                               | 9.41         | 4.00         |
| Mean                 | 9.43                               | 9.68         | 5.38         |
| Standard Deviation   | 0.575                              | 0.755        | 1.06         |
| Percent CV           | 6.10                               | 7.81         | 19.8         |
| Percent Error        | -4.98                              | -2.86        | 8.47         |

TABLE 3: RECOVERY OF ARTELINIC ACID FROM HUMAN PLASMA

| SAMPLE ID                  | SPIKED CONCENTRATION (ng/ml) | PERCENT RECOVERY | PEAK HEIGHT RATIO |               |
|----------------------------|------------------------------|------------------|-------------------|---------------|
|                            |                              |                  | SOLVENT           | PLASMA        |
| SAX CARTRIDGE              |                              |                  |                   |               |
| Low Concentration Range    |                              |                  |                   |               |
| 1                          | 19.9                         |                  | 0.122             | 0.113         |
| 2                          |                              |                  | 0.123             | 0.107         |
| 3                          |                              |                  | 0.124             | 0.076         |
| Mean (± SD)                |                              | 80.1             | 0.123 ± 0.001     | 0.098 ± 0.020 |
| Medium Concentration Range |                              |                  |                   |               |
| 1                          | 159                          |                  | 1.194             | 1.028         |
| 2                          |                              |                  | 1.147             | 1.013         |
| 3                          |                              |                  | 1.126             | 0.980         |
| Mean (± SD)                |                              | 87.1             | 1.156 ± 0.035     | 1.007 ± 0.025 |
| High Concentration Range   |                              |                  |                   |               |
| 1                          | 319                          |                  | 2.192             | 2.012         |
| 2                          |                              |                  | 2.222             | 1.898         |
| 3                          |                              |                  | 2.311             | 2.041         |
| Mean (± SD)                |                              | 88.5             | 2.242 ± 0.062     | 1.984 ± 0.076 |
| AVERAGE RECOVERY =         |                              | 85.3             |                   |               |
| NH2 CARTRIDGE              |                              |                  |                   |               |
| Low Concentration Range    |                              |                  |                   |               |
| 1                          |                              |                  | 0.052             | 0.039         |
| 2                          |                              |                  | 0.052             | 0.032         |
| 3                          |                              |                  | 0.052             | 0.033         |
| Mean (± SD)                |                              | 67.4             | 0.052 ± 0.000     | 0.035 ± 0.004 |
| Medium Concentration Range |                              |                  |                   |               |
| 1                          |                              |                  | 0.427             | 0.332         |
| 2                          |                              |                  | 0.431             | 0.318         |
| 3                          |                              |                  | 0.441             | 0.340         |
| Mean (± SD)                |                              | 76.2             | 0.433 ± 0.007     | 0.330 ± 0.011 |
| High Concentration Range   |                              |                  |                   |               |
| 1                          |                              |                  | 1.786             | 1.348         |
| 2                          |                              |                  | 1.797             | 1.340         |
| 3                          |                              |                  | 1.803             | 1.274         |
| Mean (± SD)                |                              | 73.6             | 1.795 ± 0.009     | 1.321 ± 0.041 |
| AVERAGE RECOVERY =         |                              | 72.4             |                   |               |

**TABLE 4A: INTERDAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (NH<sub>2</sub> METHOD)**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                      |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                  | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 9.50                       | 9.79                               | 9.62 | 9.79 | 8.85 | 10.1 | 9.59 | 9.62            | 0.42            | 4.36            | 1.30             |
| 38.0                       | 37.9                               | 39.5 | 37.9 | 33.9 | 35.9 | 38.2 | 37.2            | 1.99            | 5.35            | -2.06            |
| 380                        | 376                                | 340  | 396  | 365  | 338  | 320  | 356             | 28.1            | 7.91            | -6.36            |
| 760                        | 757                                | 842  | 793  | 756  | 741  | 736  | 771             | 40.2            | 5.21            | 1.43             |

**TABLE 4B: INTRADAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (NH<sub>2</sub> METHOD)**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                      |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                  | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 9.50                       | 12.1                               | 8.35 | 9.72 | 9.03 | 9.72 | 11.8 | 10.1            | 1.51            | 14.9            | 6.53             |
| 38.0                       | 34.3                               | 33.6 | 37.4 | 35.3 | 38.4 | 42.2 | 36.9            | 3.19            | 8.65            | -2.98            |
| 380                        | 369                                | 344  | 393  | 359  | 349  | 382  | 366             | 19.1            | 5.21            | -3.68            |
| 760                        | 758                                | 671  | 733  | 707  | 758  | 773  | 733             | 38.39           | 5.24            | -3.51            |

\*Measured concentrations are averages of two analyses.

**TABLE 4C: INTERDAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (SAX METHOD)**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                      |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                  | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 9.96                       | 9.15                               | 9.64 | 9.33 | 9.10 | 11.4 | 9.43 | 9.68            | 0.867           | 8.97            | -2.86            |
| 39.8                       | 42.1                               | 38.8 | 41.6 | 36.2 | 36.9 | 41.0 | 39.4            | 2.51            | 6.37            | -0.921           |
| 398                        | 401                                | 398  | 397  | 420  | 414  | 419  | 408             | 10.7            | 2.62            | 2.55             |
| 797                        | 813                                | 790  | 754  | 839  | 804  | 810  | 802             | 28.3            | 3.53            | 0.586            |

**TABLE 4D: INTRADAY PRECISION OF ARTELINIC ACID HUMAN PLASMA ASSAY (SAX METHOD)**

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                      |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                  | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 9.96                       | 9.95                               | 10.5 | 9.77 | 8.32 | 10.1 | 9.41 | 9.68            | 0.755           | 7.81            | -2.86            |
| 39.8                       | 39.5                               | 40.0 | 41.5 | 40.7 | 39.5 | 41.3 | 40.4            | 0.882           | 2.18            | 1.55             |
| 398                        | 386                                | 397  | 383  | 399  | 400  | 398  | 394             | 7.36            | 1.87            | -1.05            |
| 797                        | 789                                | 780  | 783  | 767  | 772  | 806  | 783             | 13.8            | 1.76            | -1.78            |

\*Measured concentrations are averages of two analyses.

**TABLE 5: PRECISION STANDARD CURVE CALIBRATOR STATISTICAL  
PARAMETERS FOR ARTELINIC ACID HUMAN PLASMA ASSAY**

| Standard Curve<br>Concentration<br>(ng/ml) | n | Mean<br>(ng/ml) | Standard<br>Deviation<br>(ng/ml) | Percent<br>Coefficient<br>of Variation | Percent<br>Deviation |
|--------------------------------------------|---|-----------------|----------------------------------|----------------------------------------|----------------------|
| NH2 METHOD                                 |   |                 |                                  |                                        |                      |
| 4.75                                       | 6 | 5.08            | 0.750                            | 14.8                                   | 6.84                 |
| 9.49                                       | 7 | 9.40            | 0.388                            | 4.13                                   | -0.978               |
| 19.0                                       | 7 | 19.1            | 1.32                             | 6.93                                   | 0.376                |
| 38.0                                       | 7 | 36.5            | 2.74                             | 7.51                                   | -3.95                |
| 75.9                                       | 7 | 76.6            | 1.48                             | 1.93                                   | 0.960                |
| 152                                        | 7 | 151             | 6.23                             | 4.13                                   | -0.752               |
| 304                                        | 7 | 316             | 17.3                             | 5.48                                   | 3.85                 |
| 608                                        | 6 | 600             | 61.5                             | 10.3                                   | -1.29                |
| 1220                                       | 7 | 1217            | 26.9                             | 2.21                                   | -0.234               |
| SAX METHOD                                 |   |                 |                                  |                                        |                      |
| 4.96                                       | 5 | 5.38            | 0.922                            | 17.1                                   | 8.55                 |
| 9.92                                       | 6 | 9.61            | 0.676                            | 7.04                                   | -3.18                |
| 19.8                                       | 7 | 19.8            | 1.30                             | 6.58                                   | 0.144                |
| 39.7                                       | 7 | 38.3            | 1.55                             | 4.06                                   | -3.63                |
| 79.3                                       | 7 | 80.0            | 0.796                            | 0.994                                  | 0.937                |
| 159                                        | 7 | 162             | 6.23                             | 3.85                                   | 1.80                 |
| 317                                        | 6 | 318             | 8.48                             | 2.66                                   | 0.421                |
| 635                                        | 7 | 646             | 20.0                             | 3.09                                   | 1.80                 |
| 1270                                       | 7 | 1264            | 12.7                             | 1.01                                   | -0.450               |

**TABLE 6: ACCURACY OF ARTELINIC ACID HUMAN PLASMA ASSAY  
(BLIND STUDY RESULTS) May 93**

| Sample Number | Spiked Level (ng/ml) | Measured Level (ng/ml) | Statistics (ng/ml)   |
|---------------|----------------------|------------------------|----------------------|
| 1             | 0                    | *                      | Mean =               |
| 12            |                      | *                      | SD =                 |
| 18            |                      | *                      | Percent CV =         |
| 20            |                      | *                      | Percent Bias =       |
| 2             | 23.54                | 21.6                   | Mean = 20.5          |
| 7             |                      | 21.4                   | SD = 1.23            |
| 17            |                      | 19.6                   | Percent CV = 6.00    |
| 19            |                      | 19.2                   | Percent Bias = -13.1 |
| 3             | 41.3                 | 36.3                   | Mean = 40.4          |
| 10            |                      | 37.6                   | SD = 4.48            |
| 15            |                      | 41.3                   | Percent CV = 11.1    |
| 21            |                      | 46.3                   | Percent Bias = -2.24 |
| 4             | 124.0                | 111                    | Mean = 119           |
| 11            |                      | 123                    | SD = 5.26            |
| 14            |                      | 119                    | Percent CV = 4.44    |
| 24            |                      | 121                    | Percent Bias = -4.44 |
| 5             | 200.7                | 196                    | Mean = 196           |
| 8             |                      | 200                    | SD = 3.69            |
| 13            |                      | 191                    | Percent CV = 1.88    |
| 22            |                      | 196                    | Percent Bias = -2.47 |
| 6             | 401.4                | 406                    | Mean = 404           |
| 9             |                      | 393                    | SD = 8.35            |
| 16            |                      | 413                    | Percent CV = 2.07    |
| 23            |                      | 402                    | Percent Bias = 0.523 |

#n = 3, unless a chromatogram is determined to be unacceptable.

**TABLE 7: STABILITY OF ARTELINIC ACID IN HUMAN PLASMA****Artelinic Acid Concentration of Samples Stored at -20°C**

| Spiked Concentration: | CONCENTRATION<br>(ng/ml) |      |     |     |
|-----------------------|--------------------------|------|-----|-----|
|                       | 9.50                     | 38.0 | 380 | 760 |
| TIME STORED           |                          |      |     |     |
| 0 days                | bc                       | 36.9 | 407 | 811 |
| 1 day                 | bc                       | 38.8 | 388 | 737 |
| 2 days                | bc                       | 21.0 | 180 | 640 |
| 3 days                | 8.07                     | 40.6 | 310 | 688 |
| 4 days                | 7.96                     | 39.5 | 390 | 813 |
| 1 week                | 7.92                     | 30.6 | 509 | 820 |
| 11 days               | bc                       | 46.1 | 225 | 814 |
| 2 week                | 13.8                     | 40.5 | 383 | 813 |
| 22 days               | 11.4                     | 50.5 | 414 | 868 |
| 1 month               | 11.1                     | 38.7 | 371 | 765 |
| 2 months              | 10.5                     | 39.4 | 379 | 776 |
| 3 months              | 9.68                     | 32.9 | 295 | 682 |
| 6 months              | 10.4                     | 42.4 | 392 | 717 |

**TABLE 8: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON ARTELINIC ACID SPIKED HUMAN PLASMA SAMPLES**

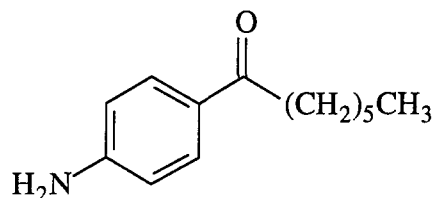
| Spiked<br>Concentration | ARTELINIC ACID |               |
|-------------------------|----------------|---------------|
|                         | Low            | High          |
|                         | Concentration  | Concentration |
| Cycle                   | (40.0 ng/ml)   | (800 ng/ml)   |
| 1                       | 35.4           | 770           |
| 2                       | 40.4           | 731           |
| 3                       | 33.9           | 676           |
| 4                       | 32.0           | 728           |
| 5                       | 38.2           | 735           |

\*\*Measured concentrations are averages of two analyses.

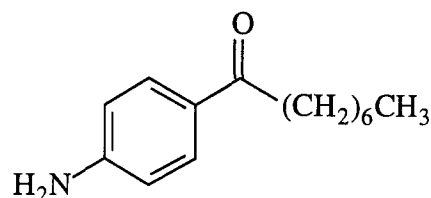
#Concentrations are mean (n = 2) results, except when chromatograms are unacceptable.

10/19/93

**ANALYTICAL STANDARD OPERATING PROCEDURE (SOP) FOR AUTOMATED HPLC  
ASSAY  
FOR *p*-AMINOHEPTANOPHENONE IN DOG PLASMA**



*p*-Aminoheptanophenone  
(WR 269,410)



Internal Standard  
*p*-Aminooctanophenone  
(WR 258,948)

**APPROVALS:**

This Analytical Standard Operating Procedure is approved for use in

Study Number: \_\_\_\_\_

INITIALS      DATE

Study Leader:      \_\_\_\_\_

QA Officer:      \_\_\_\_\_

**INSTRUMENTS:**

PUMP: LC-600 Shimadzu Pump, or equivalent.  
INJECTOR: Waters Intelligent Sample Processor Model 710 B (WISP), or equivalent.  
COLUMN: Beckman ODS 5  $\mu$ m, 25 cm X 4.6 mm, or equivalent.  
DETECTOR: Kratos Spectorflow 773, or equivalent.  
INTEGRATOR: Hewlett Packard Integrator 3392A, or equivalent.

**CONDITIONS:**

FLOW: 1.3 ml/min  
INJECTION VOLUME: 50 - 80  $\mu$ l  
RUN TIME: 22 min (PAOP (Internal Standard): 16.5 min; PAHP: 10.2 min)  
DETECTOR SETTINGS: Wavelength: 316 nm  
Absorption Range: 0.006 aufs  
Rise Time 1.0 s  
MOBILE PHASE: Acetonitrile/Water (50:50, v/v) and 0.15% H<sub>3</sub>PO<sub>4</sub>

**STANDARDS:****1. STOCK SOLUTIONS**

*p*-Aminoheptanophenone (WR 269,410)-(WRAIR, Washington, D.C.), bottle number BM 11565.

10.22 mg /100 ml in methanol

Conc. = 102 µg/ml

*p*-Aminooctanophenone Internal Standard (WR 258,948) - (WRAIR, Washington, D.C.), bottle number BM 11207.

10.5 mg /100 ml in methanol

Conc. = 105 µg/ml

**2. WORKING SOLUTIONS**

*p*-Aminoheptanophenone - Take 2.0 ml of 102 µg/ml stock and q.s. to 20 ml with methanol.

Conc. = 10.2 µg/ml

*p*-Aminoheptanophenone - Take 2.0 ml of 10.2 µg/ml working and q.s. to 20 ml with methanol.

Conc. = 1.02 µg/ml

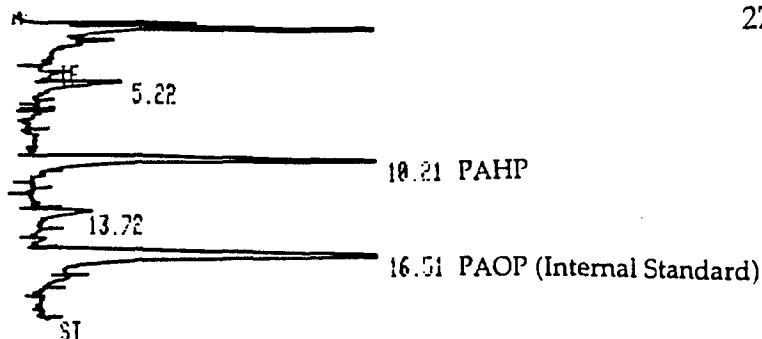
*p*-Aminooctanophenone Internal Standard - Take 1.0 ml of 105 µg/ml stock and q.s. to 1000 ml with methanol.

Conc. = 1.05 µg/ml

**SAMPLE PREPARATION:**

1. Pipet 0.5 ml of dog plasma samples into 16 X 125 culture tubes.
2. Spike standard curve samples with 00, 0, 2, 4, 8, or 15 µl of 1.02 µg/ml *p*-aminoheptanophenone working solution or 3, 6, 10, 20, or 40 µl of 10.2 µg/ml *p*-aminoheptanophenone working solution to make a standard curve. This procedure is equivalent to addition of 0, 0, 2.04, 4.08, 8.16, 15.3, 30.6, 61.2, 102, 204, and 408 ng of *p*-aminoheptanophenone, respectively, to each sample. Since 0.5 ml plasma clinical samples are assayed, these amounts correspond to 00, 0, 4.08, 8.16, 16.3, 30.6, 61.2, 122, 204, 408, and 816 ng/ml *p*-aminoheptanophenone concentrations. Vortex for 30 s.
3. Add 50 µl of 1.05 µg/ml *p*-aminooctanophenone internal standard solution. Vortex for 1 min.
4. Add 50 µl of 1N NaOH. Vortex 30 s.
5. Add 5 ml methyl *t*-butyl ether and cap tubes. Vortex 1 min. Centrifuge 10 min at 3000 g.
6. Freeze in dry ice/methanol bath. Transfer organic layer to silanized 13 X 100 culture tubes and evaporate to dryness.
7. Immediately reconstitute residue with 200 µl of mobile phase. Vortex for 1 min.
8. Transfer to WISP inserts and inject 50 - 80 µl onto column.

**COMMENTS:** Use reverse side if necessary.



RUN # 29

JUN/11/93 03:22:27

ISTD

LIST: METH 2

| RT    | HEIGHT | TYPE | CAL# | AMOUNT |
|-------|--------|------|------|--------|
| 5.22  | 2398   | BB   |      | 0.000  |
| 10.21 | 11677  | BB   | 1R   | 0.983  |
| 13.72 | 1578   | BB   |      | 0.000  |
| 16.51 | 11832  | BB   | 2L   | 1.000  |

RUN PRMTRS

ZERO = 30

ATT 2↑ = 1

CHT SP = 0.2

PK WD = 0.16

THRSH = 2

AR REJ = 0

TOTAL HGHT= 27535

ISTD AMT= 1.0000E+00

MUL FACTOR= 1.0000E+00

RPRT OPTNS

2. RF UNC PKS= 0.0000E+00

3. MUL FACTOR= 1.0000E+00

4. PK HEIGHT MODE YES

5. EXTEND RT NO

6. RPRT UNC PKS YES

TIME TBL

0.00 INTG # = 9

4.00 INTG # = -9

4.00 INTG # = 2

13.00 STOP

CALIB TBL

ISTD

CALIB RUNS 1

REF % RTW= 10.00

% RTW= 10.00

| CAL # | RT    | AMT        | AMT/HEIGHT |
|-------|-------|------------|------------|
| 1R    | 11.15 | 1.0000E+00 | 1.0000E+00 |
| 2L    | 18.10 | 1.0000E+00 | 1.0000E+00 |

WRITTEN BY:

Mila Hanz

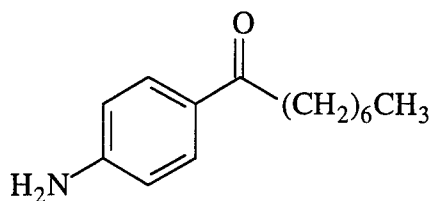
DATE: 10/31/93

NEW SOP ☒

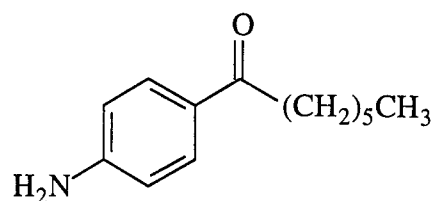
REVISION \_\_\_\_\_

10/19/93

**ANALYTICAL STANDARD OPERATING PROCEDURE (SOP) FOR AUTOMATED HPLC  
ASSAY  
FOR *p*-AMINOOCETANOPHENONE IN DOG PLASMA**



*p*-Aminooctanophenone  
(WR 258,948)



Internal Standard  
*p*-Aminoheptanophenone  
(WR 269,410)

**APPROVALS:**

This Analytical Standard Operating Procedure is approved for use in

Study Number: \_\_\_\_\_

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**INSTRUMENTS:**

PUMP: LC-600 Shimadzu Pump, or equivalent.  
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COLUMN: Beckman ODS 5  $\mu$ m, 25 cm X 4.6 mm, or equivalent.  
DETECTOR: Kratos Spectorflow 773, or equivalent.  
INTEGRATOR: Hewlett Packard Integrator 3392A, or equivalent.

**CONDITIONS:**

FLOW: 1.3 ml/min  
INJECTION VOLUME: 50 - 80  $\mu$ l  
RUN TIME: 22 min (PAOP: 18.0 min; PAHP (Internal Standard): 11.2 min)  
DETECTOR SETTINGS: Wavelength: 316 nm  
Absorption Range: 0.006 aufs  
Rise Time 1.0 s  
MOBILE PHASE: Acetonitrile/Water (50:50, v/v) and 0.15% H<sub>3</sub>PO<sub>4</sub>

**STANDARDS:**

## 1. STOCK SOLUTIONS

*p*-Aminooctanophenone (WR 258,948) - (WRAIR, Washington, D.C.), bottle number BM 11207.

10.4 mg /100 ml in methanol  
Conc. = 104 µg/ml

*p*-Aminoheptanophenone Internal Standard (WR 269,410) - (WRAIR, Washington, D.C.), bottle number BM 11565.

9.69 mg /100 ml in methanol  
Conc. = 96.9 µg/ml

## 1. WORKING SOLUTIONS

*p*-Aminooctanophenone - Take 2.0 ml of 104 µg/ml stock and q.s. to 20 ml with methanol.  
Conc. = 10.4 µg/ml

*p*-Aminooctanophenone - Take 2.0 ml of 10.4 µg/ml working and q.s. to 20 ml with methanol.

Conc. = 1.04 µg/ml

*p*-Aminoheptanophenone Internal Standard - Take 1.0 ml of 96.9 µg/ml stock solution and q.s. to 100 ml with methanol.

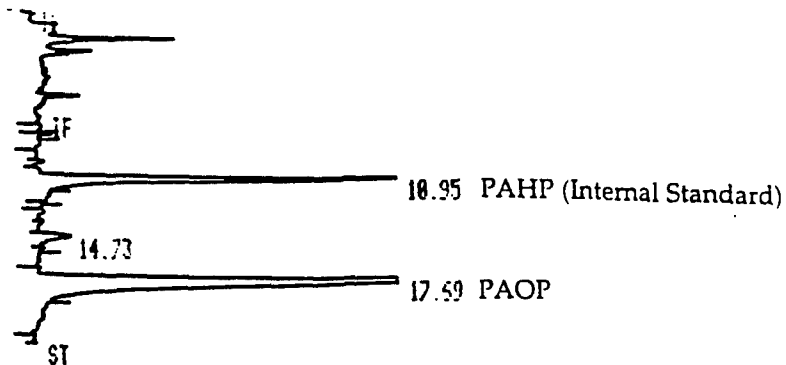
Conc. = 0.969 µg/ml

**SAMPLE PREPARATION:**

1. Pipet 0.5 ml of dog plasma samples into 16 X 125 culture tubes.
2. Spike standard curve samples with 00, 0, 2, 4, 8, or 15 µl of 1.04 µg/ml *p*-aminooctanophenone working solution or 3, 6, 10, 20, or 40 µl of 10.4 µg/ml *p*-aminooctanophenone working solution to make a standard curve. This procedure is equivalent to addition of 0, 0, 2.08, 4.16, 8.32, 15.6, 31.2, 62.4, 104, 208, and 416 ng of *p*-aminooctanophenone, respectively, to each sample. Since 0.5 ml plasma clinical samples are assayed, these amounts correspond to 00, 0, 4.16, 8.32, 16.6, 31.2, 62.4, 125, 208, 416, and 832 ng/ml *p*-aminooctanophenone concentrations. Vortex for 30 s.
3. Add 30 µl of 0.969 µg/ml *p*-aminoheptanophenone internal standard solution. Vortex for 1 min.
4. Add 50 µl of 1N NaOH. Vortex 30 s.
5. Add 5 ml methyl *t*-butyl ether and cap tubes. Vortex 1 min. Centrifuge 10 min at 3000 g.
6. Freeze in dry ice/methanol bath. Transfer organic layer to silanized 13 X 100 culture tubes and evaporate to dryness.
7. Immediately reconstitute residue with 200 µl of mobile phase. Vortex for 1 min.
8. Transfer to WISP inserts and inject 50 - 80 µl onto column.

**COMMENTS:** Use reverse side if necessary.

[AW]



RUN # 576  
 WORKFILE ID: C  
 WORKFILE NAME:

JUL/31/93 16:34:35

LIST: METH 0

ISTD

RUN PRMTRS

ZERU = 30  
 ATT 2† = 1  
 CHT SP = 0.2  
 PK WD = 0.16  
 THRSH = 2  
 AR REJ = 0

| RT    | HEIGHT | TYPE | CAL # | AMOUNT |
|-------|--------|------|-------|--------|
| 10.95 | 13088  | PB   | 1R    | 0.466  |
| 14.73 | 962    | PB   |       | 0.034  |
| 17.69 | 28117  | PB   | 2†    | 1.000  |

TOTAL HGHT= 42167

ISTD AMT= 1.0000E+00

SAMPLE AMT=

MUL FACTOR= 1.0000E+00

RPRT OPTNS

|                   |            |
|-------------------|------------|
| 2. RF UNC PKS=    | 1.0000E+00 |
| 3. MUL FACTOR=    | 1.0000E+00 |
| 4. PK HEIGHT MODE | YES        |
| 5. EXTEND RT      | NO         |
| 6. RPRT UNC PKS   | YES        |

TIME TBL

0.00 INTG # = 9  
 4.00 INTG # = 2  
 9.50 INTG # = -9  
 25.00 STOP

CALIB TBL

ISTD

CALIB RUNS 1

REF % RTM= 10.00

% RTM= 10.00

| CAL # | RT    | AMT        | AMT/HEIGHT |
|-------|-------|------------|------------|
| 1S    | 11.11 | 7.1881E+04 | 1.0000E+00 |
| 2     | 18.02 | 3.6478E+04 | 1.0000E+00 |

WRITTEN BY:

Jill Hawn

DATE:

10/22/93

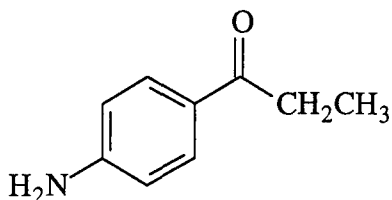
NEW SOP

✓

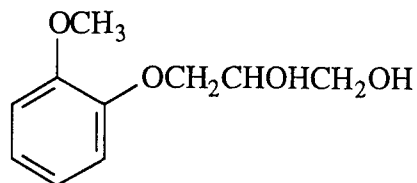
REVISION

10/19/93

ANALYTICAL STANDARD OPERATING PROCEDURE (SOP) FOR AUTOMATED HPLC  
ASSAY  
FOR *p*-AMINOPROPIOPHENONE IN DOG PLASMA



*p*-Aminopropiophenone  
(WR 000,302)



Guaifenesin  
Internal Standard

APPROVALS:

This Analytical Standard Operating Procedure is approved for use in

Study Number: \_\_\_\_\_

INITIALS      DATE

Study Leader:      \_\_\_\_\_

QA Officer:      \_\_\_\_\_

INSTRUMENTS:

PUMP: LC-600 Shimadzu Pump, or equivalent.  
INJECTOR: Waters Intelligent Sample Processor Model 710 B (WISP), or equivalent.  
COLUMN: Beckman ODS 5  $\mu$ m, 25 cm X 4.6 mm, or equivalent.  
DETECTOR: Kratos Spectorflow 773, or equivalent.  
INTEGRATOR: Hewlett Packard Integrator 3392A, or equivalent.

CONDITIONS:

FLOW: 1.0 ml/min  
INJECTION VOLUME: 40 - 80  $\mu$ l  
RUN TIME: 14 min (PAPP: 10.7 min; Guaifenesin (Internal Standard): 8.5 min)  
DETECTOR SETTINGS: Wavelength: 316 nm  
Absorption Range: 0.006 aufs  
Rise Time 1.0 s  
MOBILE PHASE: Acetonitrile/Water (20:80, v/v) and 0.15% H<sub>3</sub>PO<sub>4</sub>

**STANDARDS:**

## 1. STOCK SOLUTIONS

*p*-Aminopropiophenone (WR 000,302) - (WRAIR, Washington, D.C.), bottle number BM 11449.

10.10 mg /100 ml in methanol  
Conc. = 101 µg/ml

Guaifenesin (Internal standard) - (K & K Labs)

9.94 mg /100 ml in methanol  
Conc. = 99.4 µg/ml

## 1. WORKING SOLUTIONS

*p*-Aminopropiophenone - Take 2.0 ml of 101 µg/ml stock solution and q.s. to 20 ml with methanol.

Conc. = 10.1 µg/ml

*p*-Aminopropiophenone - Take 2.0 ml of 10.1 µg/ml working solution and q.s. to 20 ml with methanol.

Conc. = 1.01 µg/ml

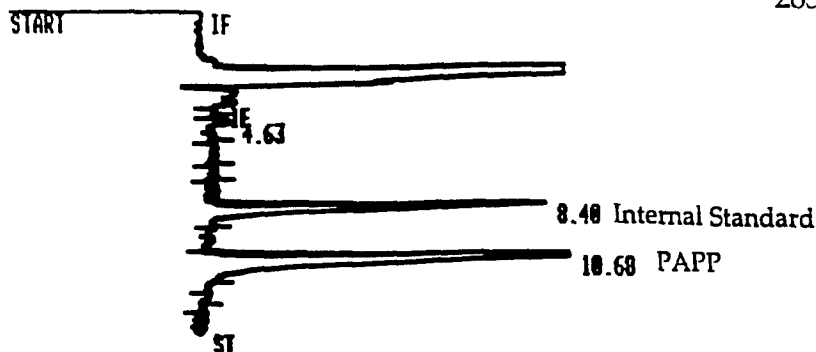
Guaifenesin (Internal standard) - Same as stock solution.

Conc. = 99.4 µg/ml

**SAMPLE PREPARATION:**

1. Pipet 0.5 ml of dog plasma samples into 16 X 125 culture tubes.
2. Spike standard curve samples with 00, 0, 2, 4, 8, or 15 µl of 1.01 µg/ml *p*-aminopropiophenone working solution or 3, 6, 10, 20, or 40 µl of 10.1 µg/ml *p*-aminopropiophenone working solution to make a standard curve. This procedure is equivalent to addition of 0, 0, 2.02, 4.04, 8.08, 15.2, 30.3, 60.6, 101, 202, and 404 ng of *p*-aminopropiophenone, respectively, to each sample. Since 0.5 ml plasma clinical samples are assayed, these amounts correspond to 00, 0, 4.04, 8.08, 16.2, 30.3, 60.6, 121, 202, 404, and 808 ng/ml *p*-aminopropiophenone concentrations. Vortex for 30 s.
3. Add 30 µl of 99.4 µg/ml guaifenesin internal standard solution. Vortex for 1 min.
4. Add 50 µl of 1N NaOH. Vortex 30 s.
5. Add 5 ml methyl *t*-butyl ether and cap tubes. Vortex 1 min, twice. Centrifuge 10 min at 3000 g.
6. Freeze in dry ice/methanol bath. Transfer organic layer to silanized 13 X 100 culture tubes and evaporate to dryness.
7. Immediately reconstitute residue with 200 µl of mobile phase. Vortex for 1 min.
8. Transfer to WISP inserts and inject 40 - 80 µl onto column.

**COMMENTS:** Use reverse side if necessary.



RUN # 252 JUN/24/93 15:00:59  
 WORKFILE ID: C  
 WORKFILE NAME:

LIST: METH 2

| RT    | HEIGHT | TYPE | CAL # | AMOUNT |
|-------|--------|------|-------|--------|
| 4.63  | 622    | BB   |       | 0.060  |
| 8.40  | 10361  | VB   | 2%    | 1.000  |
| 10.68 | 16790  | PB   | 1R    | 1.621  |

RUN PRMTRS  
 ZERO = 30  
 ATT 2+ = 1  
 CHT SP = 0.3  
 PK WD = 0.16  
 THRSH = 2  
 AR REJ = 0

TOTAL HIGHT= 27773  
 ISTD AMT= 1.0000E+00  
 SAMPLE AMT=  
 MUL FACTOR= 1.0000E+00

RPRT OPTNS  
 2. RF UNC PKS= 1.0000E+00  
 3. MUL FACTOR= 1.0000E+00  
 4. PK HEIGHT MODE YES  
 5. EXTEND RT NO  
 6. RPRT UNC PKS YES

TIME TBL  
 0.00 INTG # = 9  
 4.00 INTG # = -9  
 4.00 INTG # = 2  
 14.00 STOP

CALIB TBL  
 ISTD CALIB RUNS 1  
 REF % RTW= 10.00 % RTW= 10.00

| CAL # | RT    | AMT        | AMT/HEIGHT |
|-------|-------|------------|------------|
| 1R    | 10.70 | 1.0000E+00 | 1.0000E+00 |
| 2%    | 8.51  | 1.0000E+00 | 1.0000E+00 |

WRITTEN BY:

*Mila Stone*

DATE: 10/22/93

NEW SOP ☒

REVISION \_\_\_\_\_

**I. LABORATORY METHODOLOGY FOR WR 6026, WR 211,789 AND  
WR 254,421 (AS FREE BASES) URINE ASSAY, STUDY REPORT 22**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 712 (Waters Associates, Milford, MA), or equivalent.
2. Altex Model 100A Solvent Delivery Module (Beckman Instruments, Inc., Berkeley, CA), or equivalent.
3. Kratos Spectroflow 783 UV Detector (Kratos Analytical Instruments, Ramsey, NJ), or equivalent.
4. Hewlett-Packard Reporting Integrator #3390A (Hewlett-Packard Co., Santa Clara, CA), or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. WR 6026•2HCl - bottle no. BK 01845 (WRAIR, Washington, DC).
4. WR 211,789•2HCl•1/2H<sub>2</sub>O - bottle no. BK 50713 (WRAIR, Washington, DC).
5. WR 254,421•2HCl - bottle no. BK 18756 (WRAIR, Washington, DC).
6. Verapamil (Internal Standard) - (USP Reference Lot # F-1)
7. NaOH (Mallinckrodt, Paris, KY).
8. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).
9. Methyl-*t*-butyl ether (Baxter, Burdick & Jackson, Muskegan, MI).
10. Methanol (Fisher Scientific, Fairlawn, NJ).
11. Acetonitrile (Fisher Scientific, Fairlawn, NJ).
12. Phosphoric acid (Fisher Scientific, Fair Lawn, NJ).

\*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

### C. ASSAY CONDITIONS

#### 1. DETECTOR

##### Settings

Wavelength; 350 nm

Sensitivity; 0.005 aufs

Rise Time; 1 s

##### Lamp

ABI Analytical, Inc. (Ramsey, NJ).

#### 2. COLUMN

Axxiom Si, 5  $\mu$ m particle size, 4.6 x 250 mm - (Richard Scientific, Novato, CA).

#### 3. MOBILE PHASE

CH<sub>3</sub>CN/0.0075% H<sub>3</sub>PO<sub>4</sub> (80:20, v/v) with final apparent pH of 6.9.

#### 4. FLOW RATE

1.0 ml/min

5. STOCK SOLUTIONS - Store at 4°C, protect from light, and check for deterioration by following the ratio of drug to internal standard peak heights for a diluted solution containing both compounds (discard solutions if a more than 10% change in the ratio is observed). In any case, discard solutions within 6 months.

##### a. Precision solutions.

| Solution Type           | Weight of Standard (mg) | Purity Factor* | Preparation date: 2/9/93 |          |                         |
|-------------------------|-------------------------|----------------|--------------------------|----------|-------------------------|
|                         |                         |                | QS Volume (ml)           | Solvent  | Free Base Conc. (mg/ml) |
| WR 6026                 | 3.140                   | 0.8249*        | 10                       | 50% meOH | 0.259                   |
| WR 211,789              | 2.138                   | 0.7938**       | 10                       | 50% meOH | 0.170                   |
| WR 254,421              | 1.821                   | 0.8314***      | 10                       | 50% meOH | 0.151                   |
| Verapamil Internal Std. | 6.873                   | 1              | 6.873                    | 50% meOH | 1                       |

\*= Molecular weights of WR 6026 free base/WR 6026 • 2HCl

\*\*= Molecular weights of WR 211,789 free base/WR 211,789 • 2HCl • 1/2H<sub>2</sub>O

\*\*\*= Molecular weights of WR 254,421 free base/WR 254,421 • 2HCl

- b. Stability and blind sample analysis solutions. (WR 6026 and WR 211,789 solutions same as precision solutions.)

Preparation date: 3/26/93

| Solution Type | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent  | Free Base Conc. (µg/ml) |
|---------------|-------------------------|----------------|----------------|----------|-------------------------|
| WR 254,421    | 14.346                  | 0.8314         |                | 50% meOH | 543                     |

6. WORKING SOLUTIONS - (Store solutions at 4°C, protect from light, and discard if deterioration is observed in the stock solutions). In any case, solutions are discarded within 6 months.

- a. Combine and dilute stock precision solutions.

Preparation date: 2/9/93

| Solution Type | Conc. Diluted (mg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
|---------------|-----------------------|----------------|----------|---------------|
| WR 6026       | 0.259                 | 1:100          | 50% meOH | 2.59          |
| WR 211,789    | 0.170                 | 1.5:100        | 50% meOH | 2.55          |
| WR 254,421    | 0.151                 | 15:100         | 50% meOH | 22.7          |

- b. Combine and dilute stability and blind sample analysis solutions.

Preparation date: 3/26/93

| Solution Type | Conc. Diluted (mg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
|---------------|-----------------------|----------------|----------|---------------|
| WR 6026       | 0.259                 | 4:18.4         | 50% meOH | 56.3          |
| WR 211,789    | 0.170                 | 6:18.4         | 50% meOH | 55.4          |
| WR 254,421    | 1.19                  | 8.4:18.4       | 50% meOH | 543           |

- c. Stability and blind sample analysis solutions.

Preparation date: 3/26/93

| Solution Type | Conc. Diluted (µg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
|---------------|-----------------------|----------------|----------|---------------|
| WR 6026       | 56.3                  | 4.6:10         | 50% meOH | 25.9          |
|               | 25.9                  | 1:10           | 50% meOH | 2.59          |
| WR 211,789    | 55.4                  | 4.6:10         | 50% meOH | 25.5          |
|               | 25.5                  | 1:10           | 50% meOH | 2.55          |
| WR 254,421    | 543                   | 4.6:10         | 50% meOH | 250           |
|               | 250                   | 1:10           | 50% meOH | 25.0          |

## d. Verapamil (Internal Standard).

Preparation date: 2/9/93

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Dilution Ratio | Solvent  | Conc.<br>( $\mu\text{g}/\text{ml}$ ) |
|---------------|-------------------------------------------------|----------------|----------|--------------------------------------|
| Internal Std  | 1000                                            | 1:10           | 50% meOH | 100                                  |
| Working IS    | 100                                             | 1:2            | 50% meOH | 50                                   |

## 7. RETENTION TIMES (subject to change depending on temperature and column performance). Approximate run time: 23 min.

- a. Verapamil (Internal Standard) - 12.4 min
- b. WR 211,789 (free base) - 14.3 min
- c. WR 6026 (free base) - 15.3 min
- d. WR 254,421 (free base) - 18.2 min

## 8. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves calculated by nonweighted linear regression.

## 9. MINIMUM DETECTION LIMIT OF METHOD FOR PRECISION ANALYSIS

The minimum detection limits, 5.17 ng/ml WR 6026, 5.09 ng/ml WR 211,789, and 45.4 ng/ml WR 254,421 (as free bases) in urine, was determined as the WR 6026, WR 211,789, and WR 254,421 (as free bases) concentrations at which the signal to noise ratio was at least 3 to 1.

## 10. INJECTION VOLUME

20 to 160  $\mu\text{l}$

## 11. SAMPLE VOLUME MEASUREMENT

Pipetters used for sample volume measurement were Rainin, Gilson Pipettman and/or Eppendorf. See SOP 3-4.1 for calibration procedure.

## 12. BLANK URINE

Spiked urine samples were made with interference-free urine obtained from UCSF Department of Pharmacy staff volunteers.

## 13. WISP OPERATING TEMPERATURE

Room Temperature

#### D. SAMPLE STORAGE

Urine samples were kept frozen, if required at -70°C before analysis and thawed at room temperature for preparation and analysis.

#### E. SAMPLE PREPARATION

1. Pipette 0.5 ml urine into glass culture tube.
2. Spike precision standard curve samples with 00, 0, 1, 2, 3, 5, 10, 20, 40, or 80 µl of WR 6026, WR 211,789, and WR 254,421 standard curve working solution mixture (conc. 2.59 µg/ml of WR 6026, 2.55 µg/ml of WR 211,789 and 22.7 µg/ml of WR 254,421 as free bases) to make standard curves. This procedure is equivalent to addition of 00, 0, 2.59, 5.17, 7.76, 12.9, 25.9, 51.7, 103, or 207 ng of WR 6026, and 00, 0, 2.55, 5.09, 7.64, 12.7, 25.5, 50.9, 102, or 204 ng of WR 211,789 and 00, 0, 22.7, 45.4, 68.1, 114, 227, 454, 908, or 1820 ng of WR 254,421 to each sample. Since 0.5 ml urine clinical samples are assayed, these amounts correspond to 00, 0, 5.17, 10.3, 15.5, 25.9, 51.7, 103, 207, and 414 ng/ml WR 6026, 00, 0, 5.09, 10.2, 15.3, 25.5, 50.9, 102, 204, and 407 ng/ml WR 211,789 and 00, 0, 45.4, 90.8, 136, 227, 454, 908, 1820, and 3630 ng/ml WR 254,421 concentrations. Vortex 10 s.
3. Add 100 µl of internal standard (50 µg/ml verapamil) working solution. Vortex 10 s.
4. Add 100 µl of 1 N NaOH. Vortex 10 s.
5. Add 5 ml methyl *t*-butyl ether, cap and vortex 1 min.
6. Centrifuge 10 min at 3000 g.
7. Freeze in dry ice/methanol and transfer organic layer to a 13x100 mm culture tube.
8. Evaporate to dryness under nitrogen.
9. Reconstitute in 200 µl of mobile phase, vortex 1 min and inject 20-160 µl onto HPLC column.

#### F. QUALITY CONTROL

1. Content and frequency of blanks

\*00 = Sample without drug and without internal standard.

\*\*0 = Sample without drug and with internal standard.

No special blank was used except for the standard curve blank.

## 2. Pipette Calibration

See SOP 3-4.1.

## 3. Balance Calibration

See SOP 3-19.2.

## G. RECOVERY

Assay recovery was assessed at three different concentrations by comparing the WR 6026, WR 211,789, and WR 254,421 (as free bases) to internal standard peak height ratios in reference samples to the peak height ratios in urine. Urine and reference samples were spiked with corresponding amounts of WR 6026, WR 211,789, and WR 254,421 (as free bases). Each urine sample was prepared as described in "Sample Preparation" (Section E), except internal standard was added after the evaporation (step 8). The reference samples were spiked with WR 6026, WR 211,789, and WR 254,421 (as free bases) and with internal standard, but were not extracted and not evaporated.

## H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.5 ml urine specimens with WR 6026, WR 211,789, and WR 254,421 (as free bases) working solution as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

### Generation of Precision Samples

#### WR 6026 (as free base)

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>(ml) | Precision Sample<br>Nominal Concentration<br>(ng/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------|------------------------------------------------------|
| X-Lo | 2                              | 2.59                                               | 0.5                       | 10.4                                                 |
| Low  | 5                              | 2.59                                               | 0.5                       | 25.9                                                 |
| Med. | 30                             | 2.59                                               | 0.5                       | 155                                                  |
| Hi   | 50                             | 2.59                                               | 0.5                       | 259                                                  |

#### WR 211,789 (as free base)

|      | Volume<br>Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>(ml) | Precision Sample<br>Nominal Concentration<br>(ng/ml) |
|------|--------------------------------|----------------------------------------------------|---------------------------|------------------------------------------------------|
| X-Lo | 2                              | 2.55                                               | 0.5                       | 10.2                                                 |
| Low  | 5                              | 2.55                                               | 0.5                       | 25.5                                                 |
| Med. | 30                             | 2.55                                               | 0.5                       | 153                                                  |
| Hi   | 50                             | 2.55                                               | 0.5                       | 255                                                  |

## WR 254,421 (as free base)

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Control Volume<br>(ml) | Precision Sample Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|-------------------------------------------------|------------------------|---------------------------------------------------|
| X-Lo | 2                           | 22.7                                            | 0.5                    | 90.8                                              |
| Low  | 5                           | 22.7                                            | 0.5                    | 227                                               |
| Med. | 30                          | 22.7                                            | 0.5                    | 1360                                              |
| Hi   | 50                          | 22.7                                            | 0.5                    | 2270                                              |

## I. GENERATION OF STABILITY SAMPLES

Long term stability samples were generated by spiking rat plasma samples as shown below.

## WR 6026 (as free base)

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Control Volume<br>(ml) | Precision Sample Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|-------------------------------------------------|------------------------|---------------------------------------------------|
| X-Lo | 2                           | 2.59                                            | 0.5                    | 10.4                                              |
| Low  | 5                           | 2.59                                            | 0.5                    | 25.9                                              |
| Med. | 3                           | 25.9                                            | 0.5                    | 155                                               |
| Hi   | 5                           | 25.9                                            | 0.5                    | 259                                               |

## WR 211,789 (as free base)

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Control Volume<br>(ml) | Precision Sample Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|-------------------------------------------------|------------------------|---------------------------------------------------|
| X-Lo | 2                           | 2.55                                            | 0.5                    | 10.2                                              |
| Low  | 5                           | 2.55                                            | 0.5                    | 25.5                                              |
| Med. | 3                           | 25.5                                            | 0.5                    | 153                                               |
| Hi   | 5                           | 25.5                                            | 0.5                    | 255                                               |

## WR 254,421 (as free base)

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Control Volume<br>(ml) | Precision Sample Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|-------------------------------------------------|------------------------|---------------------------------------------------|
| X-Lo | 2                           | 25.0                                            | 0.5                    | 100                                               |
| Low  | 5                           | 25.0                                            | 0.5                    | 250                                               |
| Med. | 3                           | 250                                             | 0.5                    | 1500                                              |
| Hi   | 5                           | 250                                             | 0.5                    | 2500                                              |

Autosampler stability samples were generated by spiking 0.5 ml human urine specimens with WR 6026, WR 211,789, and WR 254,421 working solution as shown above for long term stability sample.

## J. RESULTS

## 1. STANDARD CURVE

Chromatograms for each point in a representative standard curve for WR 6026, WR 211,789, and WR 254,421 (as free bases) appear

in Figure 3. Peak height ratios for these calibrators appear in Tables 1A-C.

## 2. LOW POINT VALIDATION

Take the 6 back calculated lowest standard calibrator concentrations that were obtained in the interday precision-accuracy study as the quantitation limit interday result. Results appear in Table 2.

## 3. RECOVERY

Results for this evaluation appear in Table 3.

## 4. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 4-6.

## 5. BLIND SAMPLE ANALYSIS

Results for this evaluation appear in Tables 7A-C.

## 6. STABILITY

Results for long term stability appear in Table 8. Results for autosampler stability appear in Table 9.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
WR 6026 HUMAN URINE ASSAY  
STUDY REPORT 22

| SPIKED<br>AMOUNT<br>(ng) | STANDARD<br>CURVE         |                          | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION |
|--------------------------|---------------------------|--------------------------|---------------------------|-----------------------------|
|                          | CONCENTRATION*<br>(ng/ml) | CONCENTRATION<br>(ng/ml) |                           |                             |
| 0                        | 0                         | 0                        | 0                         | -                           |
| 2.59                     | 5.17                      | 0.050                    | 0.050                     | 3.99 <sup>a</sup>           |
| 5.17                     | 10.3                      | 0.148                    | 0.148                     | 12.2 <sup>a</sup>           |
| 7.76                     | 15.5                      | 0.190                    | 0.190                     | 15.7 <sup>a</sup>           |
| 12.9                     | 25.9                      | 0.301                    | 0.301                     | 25.0 <sup>a</sup>           |
| 25.9                     | 51.7                      | 0.620                    | 0.620                     | 51.8 <sup>a</sup>           |
| 51.7                     | 103                       | 1.358                    | 1.358                     | 126 <sup>b</sup>            |
| 103                      | 207                       | 2.060                    | 2.060                     | 194 <sup>b</sup>            |
| 207                      | 414                       | 4.357                    | 4.357                     | 414 <sup>b</sup>            |

Regression equations:

a  $y = 0.01192x + 0.0024$ ,  $r^2 = 0.9967$ ; (Low Range: 0 - 51.7 ng/ml)

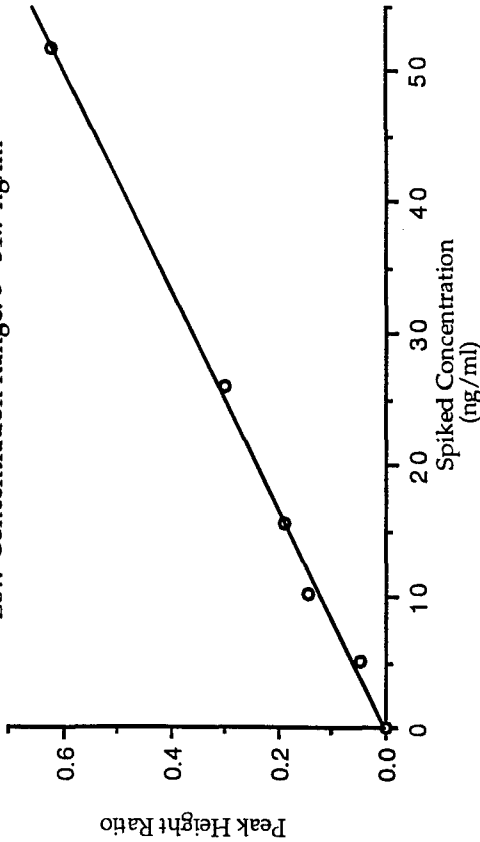
b  $y = 0.01040x + 0.0476$ ,  $r^2 = 0.9951$ ; (High Range: 0 - 414 ng/ml)

\* When 0.5 ml of biological sample is used.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 51.7 ng/ml



High Concentration Range: 0 - 414 ng/ml

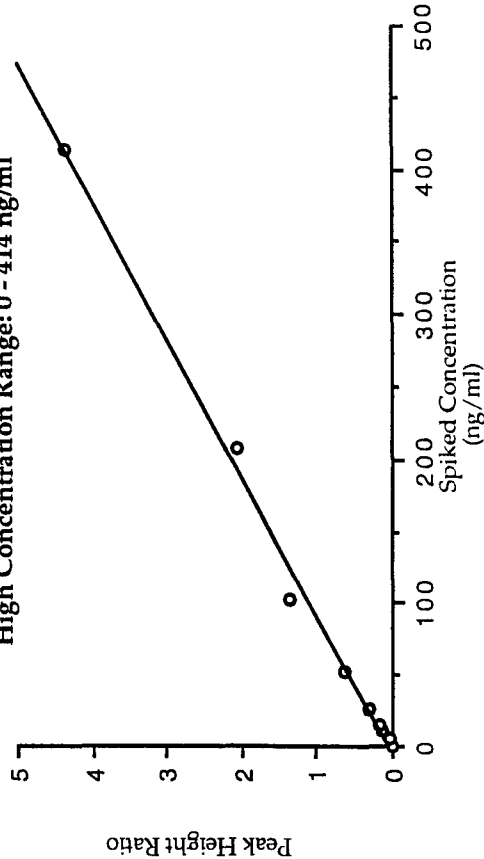


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
WR 211,789 HUMAN URINE ASSAY  
STUDY REPORT 22

| SPIKED<br>AMOUNT<br>(ng) | STANDARD<br>CURVE         |                          | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|--------------------------|---------------------------|--------------------------|---------------------------|----------------------------------------|
|                          | CONCENTRATION*<br>(ng/ml) | CONCENTRATION<br>(ng/ml) |                           |                                        |
| 0                        | 0                         | 0                        | 0                         | -                                      |
| 2.55                     | 5.09                      | 0.056                    | 0.056                     | 4.48 <sup>a</sup>                      |
| 5.09                     | 10.2                      | 0.124                    | 0.124                     | 11.3 <sup>a</sup>                      |
| 7.64                     | 15.3                      | 0.185                    | 0.185                     | 17.4 <sup>a</sup>                      |
| 12.7                     | 25.5                      | 0.253                    | 0.253                     | 24.2 <sup>a</sup>                      |
| 25.5                     | 50.9                      | 0.519                    | 0.519                     | 50.8 <sup>a</sup>                      |
| 50.9                     | 102                       | 1.185                    | 1.185                     | 117 <sup>b</sup>                       |
| 102                      | 204                       | 1.753                    | 1.753                     | 173 <sup>b</sup>                       |
| 204                      | 407                       | 4.224                    | 4.224                     | 418 <sup>b</sup>                       |

Regression equations:

a  $y = 0.01000x + 0.0112$ ,  $r^2 = 0.9948$ ; (Low Range: 0 - 50.9 ng/ml)

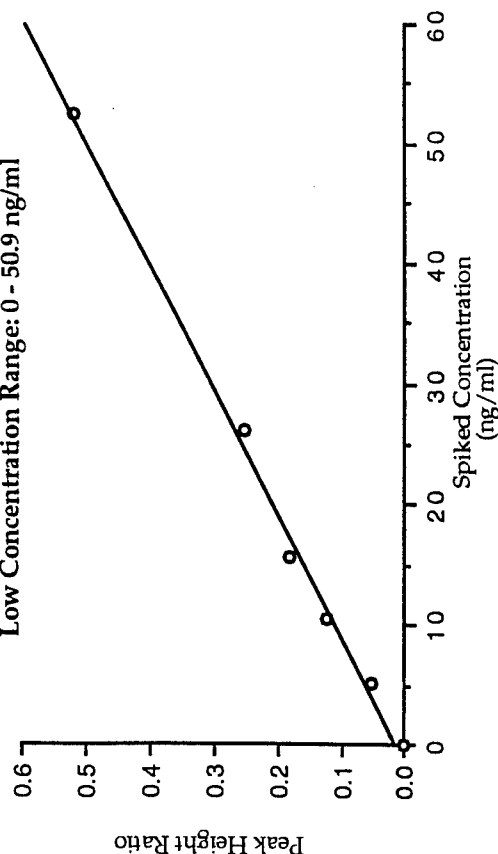
b  $y = 0.01009x + 0.0028$ ,  $r^2 = 0.9912$ ; (High Range: 0 - 407 ng/ml)

\* When 0.5 ml of biological sample is used.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 50.9 ng/ml



High Concentration Range: 0 - 407 ng/ml

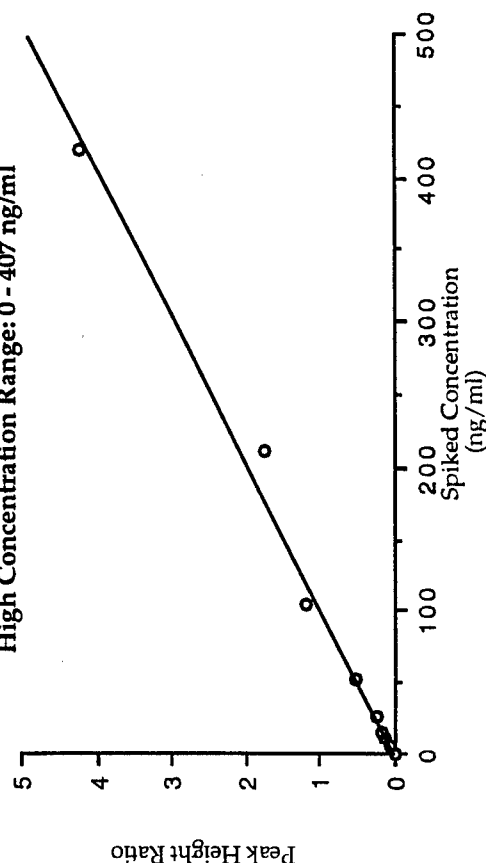


TABLE 1C: REPRESENTATIVE STANDARD CURVE FOR  
WR 254,421 HUMAN URINE ASSAY  
STUDY REPORT 22

| SPIKED<br>AMOUNT<br>(ng) | STANDARD<br>CURVE<br>CONCENTRATION*<br>(ng/ml) | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|--------------------------|------------------------------------------------|---------------------------|----------------------------------------|
| 0                        | 0                                              | 0                         | -                                      |
| 22.7                     | 45.4                                           | 0.497                     | 46.1 <sup>a</sup>                      |
| 45.4                     | 90.8                                           | 1.022                     | 98.0 <sup>a</sup>                      |
| 68.1                     | 136                                            | 1.458                     | 141 <sup>a</sup>                       |
| 114                      | 227                                            | 2.189                     | 213 <sup>a</sup>                       |
| 227                      | 454                                            | 4.664                     | 458 <sup>a</sup>                       |
| 454                      | 908                                            | 9.868                     | 1070 <sup>b</sup>                      |
| 908                      | 1820                                           | 15.346                    | 1680 <sup>b</sup>                      |
| 1820                     | 3630                                           | 32.938                    | 3650 <sup>b</sup>                      |

Regression equations:

<sup>a</sup>  $y = 0.01012x + 0.0306$ ,  $r^2 = 0.9979$ ; (Low Range: 0 - 454 ng/ml)

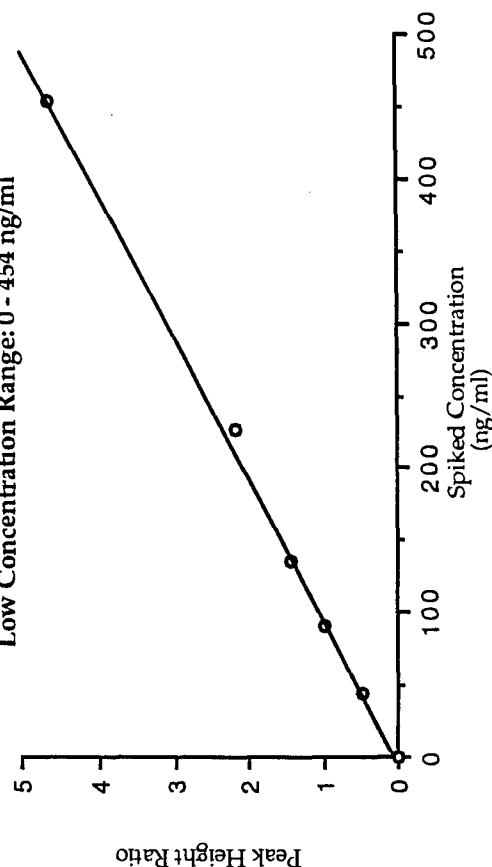
<sup>b</sup>  $y = 0.00893x + 0.2975$ ,  $r^2 = 0.9958$ ; (High Range: 0 - 3630 ng/ml)

\* When 0.5 ml of biological sample is used.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Due to the extent of the standard curve range and in order to obtain more accurate determinations of low level concentrations, two standard curves were constructed from the same set of standard curve data points.

Representative Standard Curve  
Low Concentration Range: 0 - 454 ng/ml



High Concentration Range: 0 - 3630 ng/ml

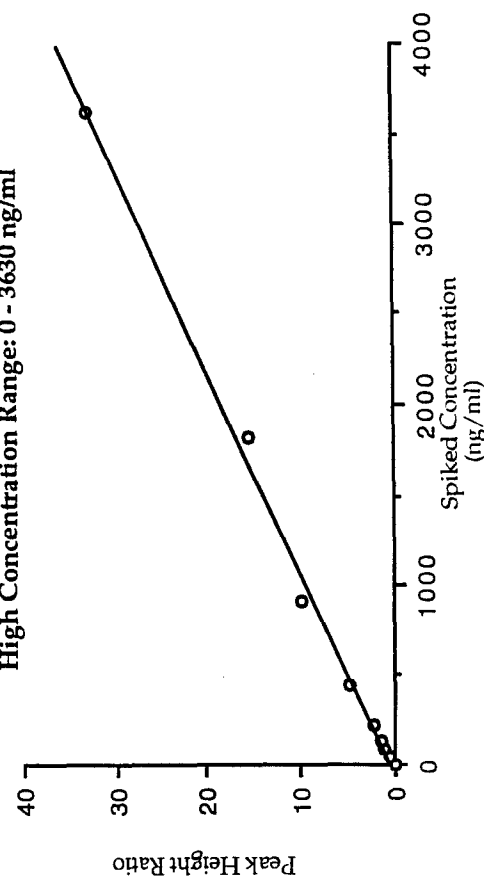


TABLE 2: WR 6026, WR 211,789, AND WR 254,421 (AS FREE BASES)  
MINIMUM QUANTITATION LIMITS

## INTERDAY

| Spiked Concentration | WR 6026<br>(5.17 ng/ml)            | WR 211,789<br>(5.09 ng/ml) | WR 254,421<br>(45.4 ng/ml) |
|----------------------|------------------------------------|----------------------------|----------------------------|
|                      | Measured Concentrations<br>(ng/ml) |                            |                            |
|                      | bc                                 | 6.51                       | 53.3                       |
|                      | 6.10                               | 4.76                       | 46.8                       |
|                      | 5.60                               | 5.88                       | 44.9                       |
|                      | 5.39                               | 5.50                       | 45.3                       |
|                      | 3.99                               | 4.48                       | 46.1                       |
|                      | 5.40                               | 4.80                       | 42.4                       |
| Mean                 | 5.30                               | 5.32                       | 46.1                       |
| Standard Deviation   | 0.785                              | 0.78                       | 3.47                       |
| Percent CV           | 14.8                               | 14.7                       | 7.51                       |
| Percent Error        | 2.44                               | 4.55                       | 1.60                       |

**TABLE 3: RECOVERIES OF WR 6026, WR 211,789 AND WR 254,421  
(AS FREE BASES) FROM HUMAN URINE**

| SAMPLE<br>ID                      | SPIKED<br>CONCENTRATION<br>Range (ng/ml) | PEAK HEIGHT RATIO |               | MEAN<br>PERCENT<br>RECOVERY |
|-----------------------------------|------------------------------------------|-------------------|---------------|-----------------------------|
|                                   |                                          | REFERENCE         | URINE         |                             |
| <u>WR 6026</u>                    |                                          |                   |               |                             |
| 1                                 | Low                                      | 0.299             | 0.291         | 91.4                        |
| 2                                 |                                          | 0.353             | 0.284         |                             |
| 3                                 |                                          | 0.340             | 0.335         |                             |
| 4                                 |                                          | 0.339             | 0.306         |                             |
| Mean (± SD)                       |                                          | 0.333 ± 0.023     | 0.304 ± 0.023 |                             |
| 1                                 | Medium                                   | 1.254             | 1.380         | 100                         |
| 2                                 |                                          | 1.519             | 1.393         |                             |
| 3                                 |                                          | 1.427             | 1.416         |                             |
| 4                                 |                                          | 1.381             | 1.394         |                             |
| Mean (± SD)                       |                                          | 1.395 ± 0.110     | 1.396 ± 0.015 |                             |
| 1                                 | High                                     | 2.153             | 2.201         | 100                         |
| 2                                 |                                          | 2.205             | 2.248         |                             |
| 3                                 |                                          | 2.204             | 2.261         |                             |
| 4                                 |                                          | 2.346             | 2.215         |                             |
| Mean (± SD)                       |                                          | 2.227 ± 0.083     | 2.231 ± 0.028 |                             |
| AVERAGE (MEAN PERCENT RECOVERY) = |                                          |                   |               | 97.2                        |

**TABLE 3: RECOVERIES OF WR 6026, WR 211,789 AND WR 254,421  
(AS FREE BASES) FROM HUMAN URINE (CONTINUED)**

| SAMPLE<br>ID                      | SPIKED<br>CONCENTRATION<br>Range (ng/ml) | PEAK HEIGHT RATIO |               | MEAN<br>PERCENT<br>RECOVERY |
|-----------------------------------|------------------------------------------|-------------------|---------------|-----------------------------|
|                                   |                                          | REFERENCE         | URINE         |                             |
| <u>WR 211,789</u>                 |                                          |                   |               |                             |
| 1                                 | Low                                      | 0.316             | 0.290         | 86.5                        |
| 2                                 |                                          | 0.334             | 0.255         |                             |
| 3                                 |                                          | 0.333             | 0.320         |                             |
| 4                                 |                                          | 0.344             | 0.283         |                             |
| Mean (± SD)                       |                                          | 0.332 ± 0.012     | 0.287 ± 0.027 |                             |
| 1                                 | Medium                                   | 1.226             | 1.315         | 96.0                        |
| 2                                 |                                          | 1.519             | 1.320         |                             |
| 3                                 |                                          | 1.421             | 1.319         |                             |
| 4                                 |                                          | 1.339             | 1.330         |                             |
| Mean (± SD)                       |                                          | 1.376 ± 0.124     | 1.321 ± 0.006 |                             |
| 1                                 | High                                     | 2.129             | 2.011         | 96.0                        |
| 2                                 |                                          | 2.158             | 2.115         |                             |
| 3                                 |                                          | 2.156             | 2.174         |                             |
| 4                                 |                                          | 2.310             | 2.100         |                             |
| Mean (± SD)                       |                                          | 2.188 ± 0.082     | 2.100 ± 0.067 |                             |
| AVERAGE (MEAN PERCENT RECOVERY) = |                                          |                   |               | 92.8                        |

**TABLE 3: RECOVERIES OF WR 6026, WR 211,789 AND WR 254,421  
(AS FREE BASES) FROM HUMAN URINE (CONTINUED)**

| SAMPLE<br>ID                      | SPIKED<br>CONCENTRATION<br>Range (ng/ml) | PEAK HEIGHT RATIO |                | MEAN<br>PERCENT<br>RECOVERY |
|-----------------------------------|------------------------------------------|-------------------|----------------|-----------------------------|
|                                   |                                          | REFERENCE         | URINE          |                             |
| <u>WR 254,421</u>                 |                                          |                   |                |                             |
| 1                                 | Low                                      | 2.422             | 2.366          | 91.8                        |
| 2                                 |                                          | 2.561             | 2.212          |                             |
| 3                                 |                                          | 2.711             | 2.512          |                             |
| 4                                 |                                          | 2.668             | 2.421          |                             |
| Mean (± SD)                       |                                          | 2.591 ± 0.129     | 2.378 ± 0.126  |                             |
| 1                                 | Medium                                   | 10.126            | 9.939          | 94.6                        |
| 2                                 |                                          | 10.942            | 10.282         |                             |
| 3                                 |                                          | 11.223            | 10.259         |                             |
| 4                                 |                                          | 10.896            | 10.388         |                             |
| Mean (± SD)                       |                                          | 10.797 ± 0.470    | 10.217 ± 0.194 |                             |
| 1                                 | High                                     | 16.446            | 16.116         | 96.2                        |
| 2                                 |                                          | 17.711            | 17.015         |                             |
| 3                                 |                                          | 17.265            | 16.684         |                             |
| 4                                 |                                          | 17.428            | 16.43          |                             |
| Mean (± SD)                       |                                          | 17.213 ± 0.543    | 16.561 ± 0.381 |                             |
| AVERAGE (MEAN PERCENT RECOVERY) = |                                          |                   |                | 94.2                        |

TABLE 4A: INTERDAY PRECISION OF WR 6026 HUMAN URINE ASSAY

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                         |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|---------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                     | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations **<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 10.4                       | 11.2                                  | 11.7 | 12.1 | 11.5 | 9.74 | 10.8 | 11.2            | 0.83            | 7.42            | 7.44             |
| 25.9                       | 23.0                                  | 25.8 | 23.2 | 24.3 | 25.3 | 22.8 | 24.1            | 1.27            | 5.28            | -7.08            |
| 155                        | 143                                   | 151  | 138  | 138  | 144  | 150  | 144             | 5.62            | 3.90            | -7.10            |
| 259                        | 255                                   | 275  | 220  | 257  | 248  | 245  | 250             | 18.0            | 7.22            | -3.47            |

TABLE 4B: INTRADAY PRECISION OF WR 6026 HUMAN URINE ASSAY

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                      |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                  | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 10.4                       | 11.9                               | 10.9 | 7.82 | 13.2 | bc   | 6.37 | 10.0            | 2.85            | 28.4            | -3.48            |
| 25.9                       | 24.5                               | 23.5 | bc   | 24.2 | 25.3 | 26.2 | 24.7            | 1.04            | 4.21            | -4.48            |
| 155                        | 152                                | 145  | 142  | 145  | 158  | 151  | 149             | 5.91            | 3.97            | -3.98            |
| 259                        | 237                                | 232  | 239  | 236  | 237  | 258  | 240             | 9.20            | 3.83            | -7.40            |

\*\* Measured concentrations are averages of two analyses.

bc = bad chromatogram.

TABLE 5A: INTERDAY PRECISION OF WR 211789 HUMAN URINE ASSAY

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                        |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|--------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                    | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations**<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 10.2                       | 9.20                                 | 11.2 | 11.1 | 11.9 | 10.2 | 11.4 | 10.8            | 0.97            | 8.98            | 6.21             |
| 25.5                       | 21.5                                 | 25.9 | 22.7 | 21.8 | 27.2 | 22.5 | 23.6            | 2.36            | 10.0            | -7.45            |
| 153                        | 141                                  | 145  | 134  | 135  | 145  | 148  | 141             | 5.75            | 4.07            | -7.63            |
| 255                        | 255                                  | 270  | 215  | 253  | 253  | 240  | 248             | 18.6            | 7.52            | -2.88            |

TABLE 5B: INTRADAY PRECISION OF WR 211789 HUMAN URINE ASSAY

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                      |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                  | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 10.2                       | 12.0                               | 12.4 | 10.9 | 7.00 | 8.89 | 7.66 | 9.81            | 2.28            | 23.3            | -3.84            |
| 25.5                       | 22.3                               | 25.5 | 20.8 | 24.7 | 27.1 | 19.1 | 23.3            | 3.04            | 13.1            | -8.82            |
| 153                        | 150                                | 146  | 143  | 155  | 136  | 137  | 145             | 7.40            | 5.12            | -5.56            |
| 255                        | 241                                | 233  | 227  | 228  | 218  | 255  | 234             | 12.9            | 5.52            | -8.37            |

\*\* Measured concentrations are averages of two analyses.

TABLE 6A: INTERDAY PRECISION OF WR 254421 HUMAN URINE ASSAY

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                        |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|--------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                    | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations**<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 90.8                       | 88.0                                 | 98.9 | 101  | 97.7 | 89.5 | 96.4 | 95.3            | 5.28            | 5.54            | 4.90             |
| 227                        | 206                                  | 238  | 217  | 208  | 215  | 212  | 216             | 11.5            | 5.34            | -4.85            |
| 1360                       | 1270                                 | 1290 | 1240 | 1230 | 1270 | 1340 | 1273            | 39.3            | 3.09            | -6.37            |
| 2270                       | 2230                                 | 2370 | 1990 | 2260 | 2180 | 2130 | 2193            | 128.5           | 5.86            | -3.38            |

TABLE 6B: INTRADAY PRECISION OF WR 254421 HUMAN URINE ASSAY

| SPIKED<br>CONC.<br>(ng/ml) | SAMPLE NUMBER                      |      |      |      |      |      | MEAN<br>(ng/ml) | S.D.<br>(ng/ml) | Percent<br>C.V. | Percent<br>Error |
|----------------------------|------------------------------------|------|------|------|------|------|-----------------|-----------------|-----------------|------------------|
|                            | 1                                  | 2    | 3    | 4    | 5    | 6    |                 |                 |                 |                  |
|                            | Measured Concentrations<br>(ng/ml) |      |      |      |      |      |                 |                 |                 |                  |
| 90.8                       | 117                                | 99.3 | 99.2 | 102  | 85.4 | 103  | 101             | 10.1            | 10.0            | 11.2             |
| 227                        | 216                                | 212  | 215  | 215  | 206  | 230  | 216             | 7.92            | 3.67            | -4.99            |
| 1360                       | 1310                               | 1270 | 1290 | 1260 | 1380 | 1260 | 1295            | 45.9            | 3.55            | -4.78            |
| 2270                       | 2060                               | 2000 | 2100 | 2040 | 2080 | 2260 | 2090            | 90.1            | 4.31            | -7.93            |

\*\* Measured concentrations are averages of two analyses.

**TABLE 7A: ACCURACY OF WR 6026 (FREE BASE) HUMAN URINE ASSAY  
(BLIND STUDY RESULTS)**

| Sample Number | Spiked Level (ng/ml) | Measured Level# (ng/ml) | Statistics (ng/ml)   |
|---------------|----------------------|-------------------------|----------------------|
| 6             | 2.60                 | *                       | Mean =               |
| 7             |                      | *                       | SD =                 |
| 16            |                      | *                       | Percent CV =         |
| 22            |                      | *                       | Percent Bias =       |
| 1             | 5.2                  | 7.12                    | Mean = 6.96          |
| 12            |                      | 7.87                    | SD = 0.732           |
| 17            |                      | 6.74                    | Percent CV = 10.5    |
| 23            |                      | 6.12                    | Percent Bias = 33.9  |
| 2             | 15.5                 | 12.5                    | Mean = 13.9          |
| 11            |                      | 15.3                    | SD = 1.32            |
| 13            |                      | 14.6                    | Percent CV = 9.51    |
| 24            |                      | 13.0                    | Percent Bias = -10.6 |
| 3             | 56.2                 | 52.2                    | Mean = 53.9          |
| 8             |                      | 52.9                    | SD = 1.63            |
| 14            |                      | 54.6                    | Percent CV = 3.03    |
| 21            |                      | 55.8                    | Percent Bias = -4.14 |
| 4             | 78.7                 | 72.3                    | Mean = 75.6          |
| 9             |                      | 76.3                    | SD = 2.32            |
| 18            |                      | 77.7                    | Percent CV = 3.07    |
| 20            |                      | 76.2                    | Percent Bias = -3.91 |
| 5             | 101.2                | 97.1                    | Mean = 97.0          |
| 10            |                      | 95.7                    | SD = 0.991           |
| 15            |                      | 98.1                    | Percent CV = 1.02    |
| 19            |                      | 97.2                    | Percent Bias = -4.13 |

# n = 3, unless a chromatogram is determined to be unacceptable.

**TABLE 7B: ACCURACY OF WR 211,789 (FREE BASE) HUMAN URINE  
ASSAY (BLIND STUDY RESULTS)**

| Sample Number | Spiked Level (ng/ml) | Measured Level# (ng/ml) | Statistics (ng/ml)   |
|---------------|----------------------|-------------------------|----------------------|
| 6             | 2.60                 | *                       | Mean =               |
| 7             |                      | *                       | SD =                 |
| 16            |                      | *                       | Percent CV =         |
| 22            |                      | *                       | Percent Bias =       |
| 1             | 5.20                 | 6.68                    | Mean = 6.97          |
| 12            |                      | 7.92                    | SD = 0.720           |
| 17            |                      | 7.04                    | Percent CV = 10.3    |
| 23            |                      | 6.22                    | Percent Bias = 33.9  |
| 2             | 15.7                 | 12.7                    | Mean = 13.9          |
| 11            |                      | 13.8                    | SD = 1.03            |
| 13            |                      | 15.2                    | Percent CV = 7.42    |
| 24            |                      | 13.7                    | Percent Bias = -11.8 |
| 3             | 57.0                 | 52.8                    | Mean = 54.8          |
| 8             |                      | 54.2                    | SD = 1.60            |
| 14            |                      | 55.7                    | Percent CV = 2.93    |
| 21            |                      | 56.4                    | Percent Bias = -3.90 |
| 4             | 79.8                 | 76.9                    | Mean = 77.2          |
| 9             |                      | 76.8                    | SD = 0.655           |
| 18            |                      | 78.2                    | Percent CV = 0.848   |
| 20            |                      | 77.0                    | Percent Bias = -3.23 |
| 5             | 102.6                | 97.1                    | Mean = 98.1          |
| 10            |                      | 97.4                    | SD = 1.94            |
| 15            |                      | 101                     | Percent CV = 1.98    |
| 19            |                      | 96.9                    | Percent Bias = -4.39 |

# n = 3, unless a chromatogram is determined to be unacceptable.

TABLE 7C: ACCURACY OF WR 254,421 (FREE BASE) HUMAN URINE  
ASSAY (BLIND STUDY RESULTS)

| Sample Number | Spiked Level (ng/ml) | Measured Level# (ng/ml) | Statistics (ng/ml)   |
|---------------|----------------------|-------------------------|----------------------|
| 6             | 5.0                  | *                       | Mean =               |
| 7             |                      | *                       | SD =                 |
| 16            |                      | *                       | Percent CV =         |
| 22            |                      | *                       | Percent Bias =       |
| 1             | 50.1                 | 56.2                    | Mean = 54.9          |
| 12            |                      | 61.9                    | SD = 5.34            |
| 17            |                      | 51.6                    | Percent CV = 9.72    |
| 23            |                      | 50.0                    | Percent Bias = 9.63  |
| 2             | 150.3                | 124                     | Mean = 134           |
| 11            |                      | 135                     | SD = 7.80            |
| 13            |                      | 135                     | Percent CV = 5.81    |
| 24            |                      | 143                     | Percent Bias = -10.7 |
| 3             | 544.1                | 513                     | Mean = 522           |
| 8             |                      | 521                     | SD = 7.85            |
| 14            |                      | 520                     | Percent CV = 1.51    |
| 21            |                      | 532                     | Percent Bias = -4.15 |
| 4             | 761.6                | 745                     | Mean = 742           |
| 9             |                      | 753                     | SD = 9.00            |
| 18            |                      | 735                     | Percent CV = 1.21    |
| 20            |                      | 734                     | Percent Bias = -2.61 |
| 5             | 979.4                | 935                     | Mean = 951           |
| 10            |                      | 948                     | SD = 13.6            |
| 15            |                      | 953                     | Percent CV = 1.43    |
| 19            |                      | 968                     | Percent Bias = -2.90 |

# n = 3, unless a chromatogram is determined to be unacceptable.

**TABLE 8: STABILITIES OF WR 6026, WR 211,789 AND WR 254,421 IN HUMAN URINE**

| WR 6026                       |                                                                        |      |     |     |
|-------------------------------|------------------------------------------------------------------------|------|-----|-----|
| Spiked Concentration (ng/ml): | 10.4                                                                   | 25.9 | 155 | 259 |
| TIME STORED                   | WR 6026 Free Base Concentration of Samples Stored at -70°C#<br>(ng/ml) |      |     |     |
| 0 days                        | 9.53                                                                   | 19.8 | 144 | 187 |
| 1 day                         | 12.2                                                                   | 25.7 | 132 | 186 |
| 2 days                        | 11.5                                                                   | 25.1 | 151 | 203 |
| 4 days                        | 9.81                                                                   | 20.9 | 137 | 185 |
| 1 week                        | 10.9                                                                   | 24.0 | 139 | 197 |
| 2 weeks                       | 9.82                                                                   | 22.0 | 131 | 218 |
| 3 weeks                       | 7.22                                                                   | 24.2 | 145 | 241 |
| 1 month                       | 8.40                                                                   | 20.5 | 160 | 239 |
| 4 months                      | 10.7                                                                   | 23.6 | 153 | 229 |

# Measured concentrations are averages of two analyses.

**TABLE 8: STABILITIES OF WR 6026, WR 211,789 AND WR 254,421 IN HUMAN URINE (Continued)**

| WR 211,789                    |                                                                           |      |     |     |
|-------------------------------|---------------------------------------------------------------------------|------|-----|-----|
| Spiked Concentration (ng/ml): | 10.2                                                                      | 25.5 | 153 | 255 |
| TIME STORED                   | WR 211,789 Free Base Concentration of Samples Stored at -70°C#<br>(ng/ml) |      |     |     |
| 0 days                        | 11.0                                                                      | 20.0 | 147 | 201 |
| 1 day                         | 12.5                                                                      | 25.5 | 130 | 194 |
| 2 days                        | 12.6                                                                      | 23.7 | 146 | 195 |
| 4 days                        | 9.78                                                                      | 20.2 | 135 | 187 |
| 1 week                        | 9.29                                                                      | 23.5 | 137 | 191 |
| 2 weeks                       | 9.47                                                                      | 22.8 | 126 | 215 |
| 3 weeks                       | 7.38                                                                      | 22.9 | 141 | 236 |
| 1 month                       | 9.77                                                                      | 19.7 | 156 | 240 |
| 4 months                      | 9.25                                                                      | 23.3 | 144 | 221 |

# Measured concentrations are averages of two analyses.

**TABLE 8: STABILITIES OF WR 6026, WR 211,789 AND WR 254,421 IN HUMAN URINE (Continued)**

WR 254,421

| Spiked Concentration (ng/ml): | 100                                                                       | 250 | 1500 | 2500 |
|-------------------------------|---------------------------------------------------------------------------|-----|------|------|
| TIME STORED                   | WR 254,421 Free Base Concentration of Samples Stored at -70°C#<br>(ng/ml) |     |      |      |
| 0 days                        | 96.0                                                                      | 205 | 1500 | 2110 |
| 1 day                         | 121                                                                       | 261 | 1470 | 2095 |
| 2 days                        | 102                                                                       | 252 | 1560 | 2070 |
| 4 days                        | 96.7                                                                      | 223 | 1440 | 1940 |
| 1 week                        | 87.3                                                                      | 226 | 1390 | 1940 |
| 2 weeks                       | 98.4                                                                      | 221 | 1310 | 2190 |
| 3 weeks                       | 96.6                                                                      | 233 | 1480 | 2400 |
| 1 month                       | 97.9                                                                      | 219 | 1570 | 2370 |
| 4 months                      | 96.2                                                                      | 232 | 1450 | 2110 |

# Measured concentrations are averages of two analyses.

TABLE 9: AUTOSAMPLER STABILITY OF WR 6026, WR 211,789 AND  
WR 254,421 IN HUMAN URINE

## WR 6026

| Spiked Concentration (ng/ml): |                                                                                       |      |     |     |
|-------------------------------|---------------------------------------------------------------------------------------|------|-----|-----|
|                               | 10.4                                                                                  | 25.9 | 155 | 259 |
| TIME STORED                   | WR 6026 Free Base Concentration of<br>Samples Stored at Room Temperature *<br>(ng/ml) |      |     |     |
| 0 hours                       | 11.1                                                                                  | 26.4 | 159 | 238 |
| 24 hours                      | 12.3                                                                                  | 25.7 | 146 | 193 |
| 48 hours                      | 14.5                                                                                  | 26.6 | 149 | 224 |

## WR 211,789

| Spiked Concentration (ng/ml): |                                                                                          |      |     |     |
|-------------------------------|------------------------------------------------------------------------------------------|------|-----|-----|
|                               | 10.2                                                                                     | 25.5 | 153 | 255 |
| TIME STORED                   | WR 211,789 Free Base Concentration of<br>Samples Stored at Room Temperature *<br>(ng/ml) |      |     |     |
| 0 hours                       | 10.5                                                                                     | 25.7 | 142 | 229 |
| 24 hours                      | 11.5                                                                                     | 24.4 | 138 | 183 |
| 48 hours                      | 11.1                                                                                     | 25.9 | 139 | 205 |

## WR 254,421

| Spiked Concentration (ng/ml): |                                                                                          |     |      |      |
|-------------------------------|------------------------------------------------------------------------------------------|-----|------|------|
|                               | 100                                                                                      | 250 | 1500 | 2500 |
| TIME STORED                   | WR 254,421 Free Base Concentration of<br>Samples Stored at Room Temperature *<br>(ng/ml) |     |      |      |
| 0 hours                       | 114                                                                                      | 250 | 1480 | 2280 |
| 24 hours                      | 112                                                                                      | 253 | 1300 | 1730 |
| 48 hours                      | 115                                                                                      | 260 | 1300 | 1900 |

\*Measured concentrations are averages of two analyses.

**LABORATORY METHODOLOGY FOR PRIMAQUINE FREE BASE AND  
CARBOXYLATED METABOLITE HUMAN PLASMA ASSAY,\* STUDY  
REPORT 23**

**A. INSTRUMENTS**

1. Refrigerated Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. Altex Model 100A Solvent Delivery Module (Beckman Instruments Inc., Berkeley, CA) or equivalent.
3. Shimadzu SPD-10AV UV Detector (Shimadzu Scientific Instruments, Columbus, MD) or equivalent.
4. Hewlett-Packard Reporting Integrator #3390A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade.
2. All chemicals are reagent grade.
3. WR 002,975AW (primaquine diphosphate) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BJ 08241, expiration date not available.
4. WR 249,725 (primaquine carboxylated metabolite) (Walter Reed Army Institute of Research, Washington D.C.), Bottle No. BM 17852, expiration date not available.
5. Mebendazole (gift from Dr. David Kirn).
6. Phosphoric acid (85%) (Fisher Scientific, Fair Lawn, NJ).
7. Acetonitrile and methanol (Fisher Scientific, Fair Lawn, NJ).
8. Tetramethylammonium Chloride (TMACl) (Fisher Scientific, Fair Lawn, NJ).
9. Ethyl Acetate (Fisher Scientific, Fair Lawn, NJ).
10. Formic acid (Sigma Chemical Co., St. Louis, MO).

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\* Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

11. Type I reagent grade water (deionized with a Nanopure II system, Barnstead Co., Boston, MA).

### C. ASSAY CONDITIONS

#### 1. DETECTOR

Settings

Wavelength: 280 nm

Range: 0.0050

#### 2. COLUMN

Axxiom ODS, 5  $\mu$ m particle size, 4.6 x 250 mm  
(Richard Scientific, Novato, CA).

#### 3. SOLVENT SYSTEM

CH<sub>3</sub>OH/CH<sub>3</sub>CN/H<sub>2</sub>O (33:18.7:48.3, v/v/v) with 0.20% TMACl and 0.13% H<sub>3</sub>PO<sub>4</sub>, pH = 5.05 (apparent pH adjusted with 85% H<sub>3</sub>PO<sub>4</sub>).

#### 4. FLOW RATE

1.0 ml/min

5. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator (in amber bottles, protected against exposure to light) and are discarded 6 months after the preparation date).

##### a. Primaquine diphosphate.

| Preparation date: 3/17/94 |                         |                |                     |          |                               |
|---------------------------|-------------------------|----------------|---------------------|----------|-------------------------------|
| Solution Type             | Weight of Standard (mg) | Purity Factor* | Solvent Volume (ml) | Solvent  | Free Base Conc. ( $\mu$ g/ml) |
| Standard Curve            | 18.702                  | 0.5696         | 18.702              | 50% MeOH | 570                           |
| Precision                 | 23.264                  | 0.5696         | 23.264              | 50% MeOH | 570                           |

\*= Molecular weights of primaquine free base/primaquine diphosphate

##### b. Carboxylated metabolite.

| Preparation date: 3/17/94 |                         |               |                     |          |                     |
|---------------------------|-------------------------|---------------|---------------------|----------|---------------------|
| Solution Type             | Weight of Standard (mg) | Purity Factor | Solvent Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
| Standard Curve            | 22.952                  | 1             | 22.952              | methanol | 1000                |
| Precision                 | 22.482                  | 1             | 22.482              | methanol | 1000                |

## c. Mebendazole (Internal Standard).

| Preparation date: 3/17/94 |                         |               |                     |                   |               |
|---------------------------|-------------------------|---------------|---------------------|-------------------|---------------|
| Solution Type             | Weight of Standard (mg) | Purity Factor | Solvent Volume (ml) | Solvent           | Conc. (µg/ml) |
| Internal Std              | 10.769                  | 1             | 10.769              | 8.12% formic acid | 1000          |

## 6. WORKING SOLUTIONS - Store solution in a 4°C refrigerator, protect against exposure to light and discard within 6 months.

## a. Primaquine diphosphate.

| Solution Type  | Conc. Diluted (µg/ml) | Dilution Ratio | Solvent  | Free Base Conc. (µg/ml) |
|----------------|-----------------------|----------------|----------|-------------------------|
| Standard Curve | 570                   | 1:10           | 50% MeOH | 57.0                    |
| Precision      | 570                   | 1:10           | 50% MeOH | 57.0                    |

## b. Carboxylated metabolite.

| Solution Type  | Conc. Diluted (µg/ml) | Dilution Ratio | Solvent  | Conc. (µg/ml) |
|----------------|-----------------------|----------------|----------|---------------|
| Standard Curve | 1000                  | 1:10           | 50% MeOH | 100           |
| Precision      | 1000                  | 1:10           | 50% MeOH | 100           |

## c. Combined primaquine diphosphate (free base concentration)/carboxylated metabolite solutions.

| Solution Type  | Conc. Diluted (µg/ml) | Dilution Ratio | Compound   | Volumes Combined (ml) | Conc. (µg/ml) |
|----------------|-----------------------|----------------|------------|-----------------------|---------------|
| Standard Curve | 57.0                  | 1:2            | primaquine | 10                    | 28.5          |
|                | 100                   | 1:2            | carboxy    | 10                    | 50.0          |
| Precision      | 57.0                  | 1:2            | primaquine | 10                    | 28.5          |
|                | 100                   | 1:2            | carboxy    | 10                    | 50.0          |

| Solution Type  | Conc. Diluted (µg/ml) | Dilution Ratio | Compound           | Solvent  | Conc. (µg/ml) |
|----------------|-----------------------|----------------|--------------------|----------|---------------|
| Standard Curve | 28.5                  | 1:10           | primaquine carboxy | 50% MeOH | 2.85          |
|                | 50.0                  |                |                    |          | 5.00          |
| Precision      | 28.5                  | 1:10           | primaquine carboxy | 50% MeOH | 2.85          |
|                | 50.0                  |                |                    |          | 5.00          |

## d. Mebendazole (Internal Standard).

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Dilution Ratio | Solvent | Conc.<br>( $\mu\text{g}/\text{ml}$ ) |
|---------------|-------------------------------------------------|----------------|---------|--------------------------------------|
| Internal Std. | 1000                                            | 3:2000         | water   | 1.50                                 |

## 7. RETENTION TIMES (subject to change depending on temperature and column performance).

- a. Primaquine as free base - 11.0 min
- b. Carboxylated metabolite - 22.5 min
- c. Mebendazole (Internal Standard) - 14.5 min

## 8. BLANK HUMAN PLASMA

Human plasma (CPD or CPDA-1 as anticoagulant) is obtained from the San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME: Samples that are expected to have high primaquine free base or carboxylated metabolite concentrations (i.e. high standard curve calibrators, high concentration control samples, and sponsor samples shown or expected to be near  $C_{\text{peak}}$ ) are injected at the low end of the volume range.

10-25  $\mu\text{l}$

## 10. QUANTITATION

By peak height ratio of drug peak and metabolite peak relative to internal standard peak. Standard curves calculated by weighted linear regression where weights =  $1/y$ .

## 11. LOWER LIMIT OF QUANTITATION OF METHOD (LLOQs were determined as the primaquine (free base) or carboxylated metabolite standard curve concentrations at which the signal to noise ratios were at least 3 to 1 and was based on the interday and intraday low point validation and on standard curve calibrator results.)

28.5 ng/ml primaquine (free base) in plasma.

20.0 ng/ml carboxylated metabolite in plasma.

## 12. SAMPLE VOLUME MEASUREMENT

Plasma sample volumes were measured with a variable volume Eppendorf pipetter.

## 13. WISP OPERATING TEMPERATURE

Refrigerated WISP temperature was less than or equal to  $10^{\circ}\text{C}$ .

14. **SAMPLE EVAPORATION:** Extracted samples are evaporated in a N-EVAP® Model 112 (Organomatic Assoc, Inc., S. Berlin, MA) by passing N<sub>2</sub> over the sample. The samples do not sit in water during evaporation.

#### D. SAMPLE STORAGE

All samples are to be kept frozen at -70°C before analysis and thawed at room temperature for preparation (within 30 min) and analysis, unless otherwise specified.

#### E. SAMPLE PREPARATION

1. If frozen, thaw human plasma sample at room temperature and vortex for 1 min. Pipet 0.500 ml of human plasma sample into a screw cap glass culture tube.
2. Spike standard curve samples with 00,\* 0,\*\* 0.2, 0.5, 1, 2, 3, 4, 5, 8, 15, 25, or 50 µl of 28.5 µg/ml primaquine diphosphate (free base concentration) and 50.0 µg/ml carboxylated metabolite working solution to make standard curves. Since 0.500 ml plasma samples are assayed, this procedure is equivalent to making standard curve samples with primaquine free base concentrations corresponding to 00, 0, 28.5, 57.0, 114, 171, 228, 285, 456, 854, 1420, and 2850 ng/ml and carboxylated metabolite concentrations corresponding to 00, 0, 20.0, 50.0, 100, 200, 300, 400, 500, 800, 1500, 2500, and 5000 ng/ml. Vortex 10 s.
3. Add 100 µl of internal standard (mebendazole = 1.50 µg/ml) solution. Vortex 10 s.
4. Add 3.0 ml of ethyl acetate.
5. Cap tube, and vortex 30 s, twice at speed 5 (VWR Multitube Vortexer). Centrifuge at 3000 g for 10 min.
6. Freeze aqueous layer in a dry ice/methanol bath and transfer organic phase into a 12x75 mm culture tube. Evaporate organic layer to dryness under nitrogen.
7. Add 2.0 ml of ethyl acetate to the aqueous layer. Repeat steps 5 and 6.
8. Reconstitute with 100 µl of 40% acetonitrile solution, vortex 1 min, and centrifuge at 3000 g for 10-15 min.
9. Transfer to WISP vial and inject 10-25 µl onto HPLC column.

---

\* 00 = Sample with no drug and no internal standard.

\*\* 0 = Sample with no drug but with internal standard.

## F. QUALITY CONTROL

### 1. Content and frequency of blanks

No special blank was used except for the standard curve blank.

### 2. Pipette Calibration

See ASOP 2C-1.1.

### 3. Balance Calibration

See ASOP 2C-2.1.

## G. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the primaquine (as free base) and carboxylated metabolite to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.5 ml) samples were spiked with primaquine (as free base) and carboxylated metabolite, then prepared as described above in "Sample Preparation" steps 3-9, except that in step 6; the sample was not evaporated to dryness and after step 7; 4 ml of ethyl acetate was taken, the internal standard was added, the samples vortexed 30 s, and the sample was evaporated to dryness. Reference samples were generated by spiking 5 ml of ethyl acetate with drug and metabolite to correspond with plasma samples, removing 4 ml of the resulting solutions, adding internal standard, evaporating to dryness then following steps 8 and 9 of "Sample Preparation."

## H. GENERATION OF PRECISION SAMPLES

Samples for precision analysis were generated by spiking 0.5 ml plasma specimens with primaquine (as free base) and carboxylated metabolite working solutions as shown below. These samples were then prepared as described in Sample Preparation (Section E) above.

Generation of Precision Samples

|      | Volume<br>Spiked<br>( $\mu$ l) | Primaquine<br>Free Base<br>Spiking<br>Solution<br>Concentration<br>( $\mu$ g/ml) | Carboxylated<br>Metabolite<br>Spiking<br>Solution<br>Concentration<br>( $\mu$ g/ml) | Plasma<br>Volume<br>(ml) | Nominal<br>Primaquine<br>Free Base<br>Concentration<br>(ng/ml) | Nominal<br>Carboxylated<br>Metabolite<br>Concentration<br>(ng/ml) |
|------|--------------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------|----------------------------------------------------------------|-------------------------------------------------------------------|
| X-Lo | 1.5                            | 28.5                                                                             | 50.0                                                                                | 0.5                      | 85.4                                                           | 150                                                               |
| Low  | 3                              | 28.5                                                                             | 50.0                                                                                | 0.5                      | 171                                                            | 300                                                               |
| Med. | 8                              | 28.5                                                                             | 50.0                                                                                | 0.5                      | 456                                                            | 800                                                               |
| Hi   | 25                             | 28.5                                                                             | 50.0                                                                                | 0.5                      | 1420                                                           | 2500                                                              |

## I. GENERATION OF STABILITY SAMPLES

1. Long term stability samples were generated by spiking pooled human plasma samples as shown below.

|                   | Volume Spiked (μl) | Primaquine Free Base Spiking Solution Concentration (μg/ml) | Carboxylated Metabolite Spiking Solution Concentration (μg/ml) | Plasma Volume (ml) | Nominal Primaquine Free Base Concentration (ng/ml) | Nominal Carboxylated Metabolite Concentration (ng/ml) |
|-------------------|--------------------|-------------------------------------------------------------|----------------------------------------------------------------|--------------------|----------------------------------------------------|-------------------------------------------------------|
| For -20°C Samples |                    |                                                             |                                                                |                    |                                                    |                                                       |
| X-Lo              | 45                 | 28.5                                                        | 50.0                                                           | 14.955             | 85.4                                               | 150                                                   |
| Low               | 90                 | 28.5                                                        | 50.0                                                           | 14.910             | 171                                                | 300                                                   |
| Med.              | 240                | 28.5                                                        | 50.0                                                           | 14.760             | 456                                                | 800                                                   |
| Hi                | 750                | 28.5                                                        | 50.0                                                           | 14.250             | 1420                                               | 2500                                                  |
| For -70°C Samples |                    |                                                             |                                                                |                    |                                                    |                                                       |
| X-Lo              | 30                 | 28.5                                                        | 50.0                                                           | 9.970              | 85.4                                               | 150                                                   |
| Low               | 60                 | 28.5                                                        | 50.0                                                           | 9.940              | 171                                                | 300                                                   |
| Med.              | 160                | 28.5                                                        | 50.0                                                           | 9.840              | 456                                                | 800                                                   |
| Hi                | 500                | 28.5                                                        | 50.0                                                           | 9.500              | 1420                                               | 2500                                                  |

2. Benchtop stability samples were generated by spiking pooled human plasma samples as shown below.

|      | Volume Spiked (μl) | Primaquine Free Base Spiking Solution Concentration (μg/ml) | Carboxylated Metabolite Spiking Solution Concentration (μg/ml) | Plasma Volume (ml) | Nominal Primaquine Free Base Concentration (ng/ml) | Nominal Carboxylated Metabolite Concentration (ng/ml) |
|------|--------------------|-------------------------------------------------------------|----------------------------------------------------------------|--------------------|----------------------------------------------------|-------------------------------------------------------|
| X-Lo | 15                 | 28.5                                                        | 50.0                                                           | 4.985              | 85.4                                               | 150                                                   |
| Low  | 30                 | 28.5                                                        | 50.0                                                           | 4.97               | 171                                                | 300                                                   |
| Med. | 80                 | 28.5                                                        | 50.0                                                           | 4.92               | 456                                                | 800                                                   |
| Hi   | 250                | 28.5                                                        | 50.0                                                           | 4.75               | 1420                                               | 2500                                                  |

3. System stability samples were generated by spiking 0.5 ml human plasma specimens with primaquine (as free base) and carboxylated metabolite working solution as shown in Section H, "Generation of Precision Samples."
4. The effect of repeated freeze and thaw cycles on stability of primaquine (as free base) and carboxylated metabolite in human plasma samples was determined as described in laboratory SOP 2D-3.1, Section G.2. Freeze/thaw samples at Hi and Low concentrations were generated as described above for precision samples and in laboratory SOP 2D-3.1, Section G.2.

## J. RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for primaquine (as free base) and carboxylated metabolite appear in Figure 3. Peak height ratios and corresponding standard curve concentrations for these calibrators appear in Table 1.

### 2. STUDY STANDARD CURVE CALIBRATOR STATISTICAL PARAMETERS

Results for this evaluation appears in Table 2

### 3. LOW POINT VALIDATION

The 6 back calculated lowest standard calibrator concentrations that were obtained in the interday precision-accuracy study were used as the interday minimum quantitation limit data. Results obtained for 6 lowest standard calibrator samples run with a standard curve were used as the intraday minimum quantitation limit data. Results appear in Table 3.

### 4. INTRA- AND INTERDAY PRECISION

Results for precision samples generated as described in Section H above were used for evaluations that appear in Tables 2 and 3.

### 5. RECOVERY

Results for recovery samples generated as described in Section G above were used for the evaluation that appears in Table 5.

### 6. STABILITY

- a. Long term stability results appear in Tables 7 and 8.
- b. Bench top stability results appear in Table 9.
- c. System (prepared sample) stability results appear in Table 10.
- d. Freeze/thaw stability results appear in Table 11.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
PRIMAQUINE FREE BASE IN HUMAN PLASMA  
ASSAY, STUDY REPORT 23

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) |       |                           |                                        |
|                           | 0                        | 0     | 0                         | *                                      |
| 14.2                      | 28.5                     | 0.052 |                           | 33.0                                   |
| 28.5                      | 57.0                     | 0.101 |                           | 59.5                                   |
| 57.0                      | 114                      | 0.202 |                           | 114                                    |
| 85.4                      | 171                      | 0.292 |                           | 163                                    |
| 114                       | 228                      | 0.392 |                           | 217                                    |
| 142                       | 285                      | 0.503 |                           | 277                                    |
| 228                       | 456                      | 0.806 |                           | 441                                    |
| 427                       | 854                      | 1.483 |                           | 807                                    |
| 712                       | 1420                     | 2.570 |                           | 1390                                   |
| 1420                      | 2850                     | 5.486 |                           | 2970                                   |

Regression equations:\*\*\*

$$y = 0.001850x - 0.009103, r^2 = 0.9979$$

\* Into 0.5 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression, where weight =  $1/y$ .

Representative Standard Curve

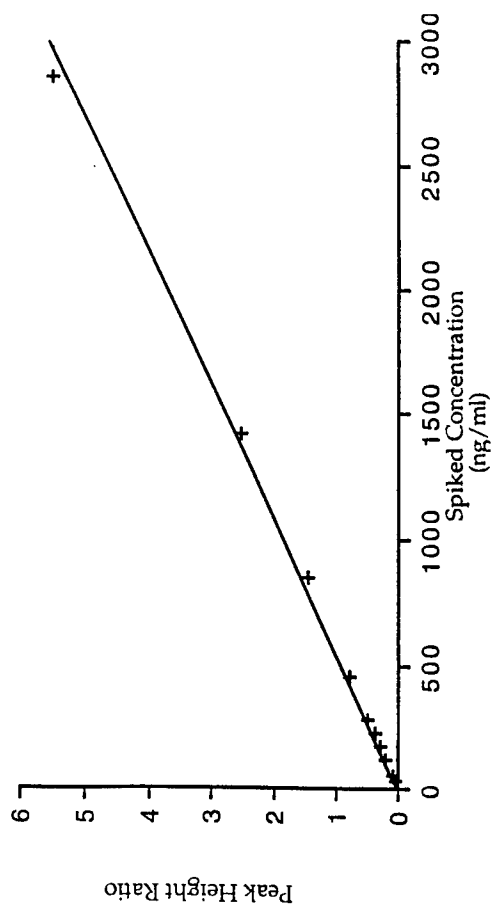


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR CARBOXY-PRIMAQUINE METABOLITE IN HUMAN PLASMA ASSAY, STUDY REPORT 23

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) |       |                           |                                        |
|                           | 0                        | 0     |                           | *                                      |
| 10.0                      | 20.0                     | 0.035 |                           | 23.2                                   |
| 25.0                      | 50.0                     | 0.067 |                           | 48.9                                   |
| 50.0                      | 100                      | 0.127 |                           | 97.2                                   |
| 100                       | 200                      | 0.264 |                           | 207                                    |
| 150                       | 300                      | 0.373 |                           | 295                                    |
| 200                       | 400                      | 0.480 |                           | 381                                    |
| 250                       | 500                      | 0.620 |                           | 494                                    |
| 400                       | 800                      | 0.996 |                           | 796                                    |
| 750                       | 1500                     | 1.809 |                           | 1450                                   |
| 1250                      | 2500                     | 3.121 |                           | 2510                                   |
| 2500                      | 5000                     | 6.316 |                           | 5080                                   |

Regression equations:

$$y = 0.001243x + 0.006200, r^2 = 0.9995$$

\* Into 0.5 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression, where weight =  $1/y$ .

Representative Standard Curve

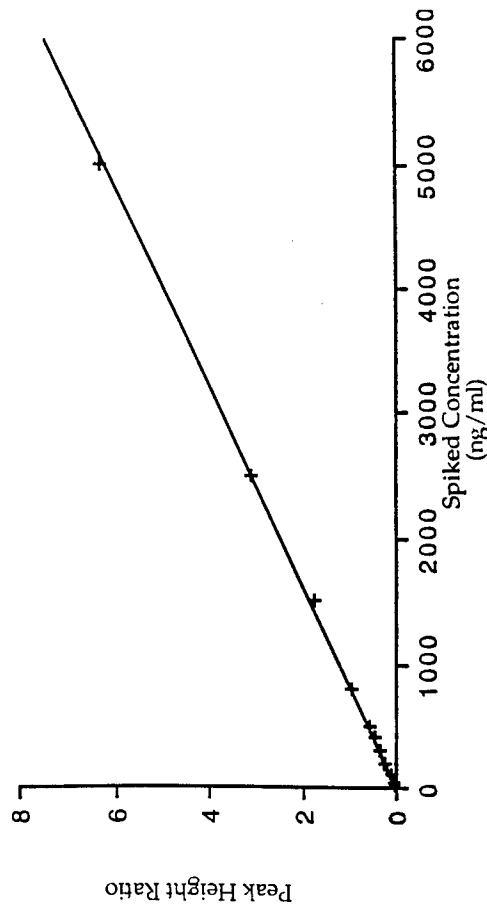


TABLE 2: PRECISION STANDARD CURVE CALIBRATOR STATISTICAL  
PARAMETERS FOR PRIMAQUINE HUMAN PLASMA ASSAY

| Spiked<br>Concentration<br>(ng/ml) | n | Mean<br>(ng/ml) | Standard<br>Deviation<br>(ng/ml) | Percent<br>C.V. | Percent<br>R.E. |
|------------------------------------|---|-----------------|----------------------------------|-----------------|-----------------|
| Primaquine Free Base               |   |                 |                                  |                 |                 |
| 28.5                               | 7 | 33.2            | 2.11                             | 6.34            | 16.6            |
| 57                                 | 7 | 58.1            | 2.74                             | 4.73            | 1.85            |
| 114                                | 7 | 112             | 4.38                             | 3.90            | -1.63           |
| 171                                | 7 | 164             | 3.26                             | 1.99            | -4.34           |
| 228                                | 7 | 219             | 8.08                             | 3.68            | -3.76           |
| 285                                | 7 | 271             | 8.50                             | 3.13            | -4.76           |
| 456                                | 7 | 440             | 13.4                             | 3.04            | -3.48           |
| 854                                | 7 | 829             | 20.4                             | 2.46            | -2.96           |
| 1420                               | 7 | 1420            | 32.0                             | 2.26            | -0.20           |
| 2850                               | 7 | 2930            | 71.3                             | 2.43            | 2.76            |
| Carboxylated Metabolite            |   |                 |                                  |                 |                 |
| 20                                 | 7 | 19.7            | 2.92                             | 14.8            | -1.50           |
| 50                                 | 7 | 49.8            | 3.45                             | 6.93            | -0.37           |
| 100                                | 7 | 101             | 3.96                             | 3.92            | 1.01            |
| 200                                | 7 | 205             | 7.26                             | 3.54            | 2.50            |
| 300                                | 7 | 300             | 8.59                             | 2.87            | -0.048          |
| 400                                | 7 | 405             | 15.2                             | 3.76            | 1.14            |
| 500                                | 7 | 505             | 14.5                             | 2.87            | 1.00            |
| 800                                | 7 | 809             | 21.8                             | 2.69            | 1.07            |
| 1500                               | 7 | 1460            | 66.2                             | 4.54            | -2.76           |
| 2500                               | 7 | 2470            | 25.0                             | 1.01            | -1.09           |
| 5000                               | 7 | 5050            | 56.8                             | 1.12            | 1.06            |

TABLE 3: LOWER LIMITS OF QUANTITATION OF THE PRIMAQUINE  
HUMAN PLASMA ASSAY

Primaquine Free Base

| Spiked Concentration | (28.5 ng/ml)                       | (28.5 ng/ml) |
|----------------------|------------------------------------|--------------|
| Sample               | Measured Concentrations<br>(ng/ml) |              |
|                      | Interday                           | Intraday     |
| 1                    | 33.6                               | 34.9         |
| 2                    | 31.3                               | 35.5         |
| 3                    | 35.1                               | 30.3         |
| 4                    | 35.6                               | 32.6         |
| 5                    | 33.0                               | 34.3         |
| 6                    | 34.3                               | 35.5         |
| Mean                 | 33.8                               | 33.9         |
| Standard Deviation   | 1.56                               | 2.05         |
| Percent CV           | 4.60                               | 6.04         |
| Percent R.E.         | 18.7                               | 18.8         |

Carboxylated Metabolite

| Spiked Concentration | (20.0 ng/ml)                       | (20.0 ng/ml) |
|----------------------|------------------------------------|--------------|
| Sample               | Measured Concentrations<br>(ng/ml) |              |
|                      | Interday                           | Intraday     |
| 1                    | 20.4                               | 20.0         |
| 2                    | 18.9                               | 18.5         |
| 3                    | 16.0                               | bc           |
| 4                    | 16.1                               | 18.5         |
| 5                    | 23.2                               | 17.0         |
| 6                    | 23.0                               | 16.3         |
| Mean                 | 19.6                               | 18.1         |
| Standard Deviation   | 3.19                               | 1.45         |
| Percent CV           | 16.3                               | 8.01         |
| Percent R.E.         | -2.00                              | -9.70        |

TABLE 4: PRECISION OF PRIMAQUINE HUMAN PLASMA ASSAY

Interday Precision Primaquine

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 85.4                          | 171   | 456   | 1420  |
| Measured Concentrations (ng/mL) |                  |                               |       |       |       |
| 1                               | 1                | 85.8                          | 157   | 442   | 1320  |
|                                 | 2                | 85.8                          | 157   | 446   | 1420  |
| 2                               | 1                | 81                            | 166   | 443   | 1400  |
|                                 | 2                | 85.3                          | 173   | 436   | 1380  |
| 3                               | 1                | 82.2                          | 158   | 426   | 1390  |
|                                 | 2                | 82.2                          | 164   | 426   | 1390  |
| 4                               | 1                | 91.5                          | 167   | 441   | 1450  |
|                                 | 2                | 81.5                          | 161   | 426   | 1340  |
| 5                               | 1                | 87.1                          | 163   | 448   | 1410  |
|                                 | 2                | 82.2                          | 162   | 444   | 1390  |
| 6                               | 1                | 83.4                          | 161   | 459   | 1440  |
|                                 | 2                | 82.8                          | 160   | 431   | 1400  |
| n                               |                  | 12                            | 12    | 12    | 12    |
| Mean                            |                  | 84.2                          | 162   | 439   | 1390  |
| S.D.                            |                  | 3.02                          | 4.64  | 10.3  | 36.8  |
| Percent C.V.                    |                  | 3.58                          | 2.86  | 2.34  | 2.64  |
| Percent R.E.                    |                  | -1.37                         | -5.02 | -3.73 | -1.82 |

Intraday Precision Primaquine

| Validation                      | QC         | Spiked Concentrations (ng/mL) |       |       |      |
|---------------------------------|------------|-------------------------------|-------|-------|------|
| Run No.                         | Sample No. | 85.4                          | 171   | 456   | 1420 |
| Measured Concentrations (ng/mL) |            |                               |       |       |      |
| 7                               | 1          | 84.4                          | 166   | 453   | 1440 |
|                                 | 2          | 83.1                          | 159   | 437   | 1460 |
|                                 | 3          | 81.9                          | 164   | 446   | 1410 |
|                                 | 4          | 83.8                          | 165   | 453   | 1400 |
|                                 | 5          | 84.4                          | 160   | 458   | 1440 |
|                                 | 6          | 85.0                          | 176   | 434   | 1460 |
| n                               |            | 6                             | 6     | 6     | 6    |
| Mean                            |            | 83.8                          | 165   | 447   | 1435 |
| S.D.                            |            | 1.12                          | 6.07  | 9.62  | 25.1 |
| Percent C.V.                    |            | 1.34                          | 3.68  | 2.15  | 1.75 |
| Percent R.E.                    |            | -1.91                         | -3.51 | -2.01 | 1.06 |

TABLE 5: PRECISION OF PRIMAQUINE HUMAN PLASMA ASSAY

Interday Precision Carboxy-Primaquine

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |        |      |        |
|---------------------------------|------------------|-------------------------------|--------|------|--------|
|                                 |                  | 150                           | 300    | 800  | 2500   |
| Measured Concentrations (ng/mL) |                  |                               |        |      |        |
| 1                               | 1                | 149                           | 292    | 804  | 2490   |
|                                 | 2                | 160                           | 306    | 806  | 2540   |
| 2                               | 1                | 161                           | 298    | 816  | 2540   |
|                                 | 2                | 157                           | 316    | 791  | 2550   |
| 3                               | 1                | 153                           | 307    | 847  | 2660   |
|                                 | 2                | 151                           | 314    | 867  | 2570   |
| 4                               | 1                | 158                           | 298    | 824  | 2460   |
|                                 | 2                | 157                           | 289    | 816  | 2340   |
| 5                               | 1                | 146                           | 288    | 818  | 2480   |
|                                 | 2                | 153                           | 298    | 793  | 2450   |
| 6                               | 1                | 139                           | 296    | 810  | 2500   |
|                                 | 2                | 141                           | 288    | 763  | 2440   |
| n                               |                  | 12                            | 12     | 12   | 12     |
| Mean                            |                  | 152                           | 299    | 813  | 2500   |
| S.D.                            |                  | 7.19                          | 9.68   | 26.6 | 79.8   |
| Percent C.V.                    |                  | 4.73                          | 3.23   | 3.27 | 3.19   |
| Percent R.E.                    |                  | 1.39                          | -0.278 | 1.61 | 0.0667 |

Intraday Precision Carboxy-Primaquine

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |      |      |       |
|---------------------------------|------------------|-------------------------------|------|------|-------|
|                                 |                  | 150                           | 300  | 800  | 2500  |
| Measured Concentrations (ng/mL) |                  |                               |      |      |       |
| 7                               | 1                | 152                           | 294  | 789  | 2220  |
|                                 | 2                | 153                           | 301  | 733  | 2440  |
|                                 | 3                | 147                           | 309  | 794  | 2320  |
|                                 | 4                | 152                           | 328  | 806  | 2330  |
|                                 | 5                | 136                           | 324  | 834  | 2470  |
|                                 | 6                | 159                           | 320  | 760  | 2610  |
| n                               |                  | 6                             | 6    | 6    | 6     |
| Mean                            |                  | 149                           | 324  | 800  | 2470  |
| S.D.                            |                  | 11.8                          | 4.00 | 37.4 | 140   |
| Percent C.V.                    |                  | 7.91                          | 1.23 | 4.67 | 5.67  |
| Percent R.E.                    |                  | -0.67                         | 8.00 | 0.00 | -1.20 |

TABLE 6: RECOVERIES OF PRIMAQUINE AND CARBOXYLATED METABOLITE FROM HUMAN PLASMA

| SAMPLE ID                      | SPIKED CONCENTRATION<br>Range (ng/ml) |      | PEAK HEIGHT RATIO |              | AVERAGE PERCENT RECOVERY |
|--------------------------------|---------------------------------------|------|-------------------|--------------|--------------------------|
|                                |                                       |      | REFERENCE         | PLASMA       |                          |
| <u>Primaquine</u>              |                                       |      |                   |              |                          |
| 1                              | High                                  | 1420 | 5.800             | 5.821        | 94.6                     |
| 2                              |                                       |      | 5.969             | 5.639        |                          |
| 3                              |                                       |      | 6.423             | 5.750        |                          |
| Mean (± SD)                    |                                       |      | 6.064 ±0.322      | 5.737 ±0.092 |                          |
| 1                              | Medium                                | 456  | 1.798             | 1.776        | 97.7                     |
| 2                              |                                       |      | 1.782             | 1.780        |                          |
| 3                              |                                       |      | 1.862             | 1.763        |                          |
| Mean (± SD)                    |                                       |      | 1.814 ±0.042      | 1.773 ±0.009 |                          |
| 1                              | Low                                   | 171  | 0.629             | 0.659        | 104.0                    |
| 2                              |                                       |      | 0.628             | 0.667        |                          |
| 3                              |                                       |      | 0.639             | 0.645        |                          |
| Mean (± SD)                    |                                       |      | 0.632 ±0.006      | 0.657 ±0.011 |                          |
| 1                              | X Low                                 | 85.4 | 0.293             | 0.306        | 107.4                    |
| 2                              |                                       |      | 0.305             | 0.317        |                          |
| 3                              |                                       |      | 0.277             | 0.317        |                          |
| Mean (± SD)                    |                                       |      | 0.292 ±0.014      | 0.313 ±0.006 |                          |
| OVERALL AVERAGE RECOVERY =     |                                       |      |                   |              | 100.9                    |
| <u>Carboxylated metabolite</u> |                                       |      |                   |              |                          |
| 1                              | High                                  | 2500 | 8.878             | 6.681        | 72.9                     |
| 2                              |                                       |      | 8.915             | 6.645        |                          |
| 3                              |                                       |      | 9.604             | 6.653        |                          |
| Mean (± SD)                    |                                       |      | 9.132 ±0.409      | 6.660 ±0.019 |                          |
| 1                              | Medium                                | 800  | 2.851             | 2.128        | 73.6                     |
| 2                              |                                       |      | 2.849             | 2.089        |                          |
| 3                              |                                       |      | 2.851             | 2.078        |                          |
| Mean (± SD)                    |                                       |      | 2.850 ±0.001      | 2.098 ±0.026 |                          |
| 1                              | Low                                   | 300  | 1.073             | 0.840        | 76.8                     |
| 2                              |                                       |      | 1.071             | 0.840        |                          |
| 3                              |                                       |      | 1.069             | 0.786        |                          |
| Mean (± SD)                    |                                       |      | 1.071 ±0.002      | 0.822 ±0.031 |                          |
| 1                              | X Low                                 | 150  | 0.532             | 0.393        | 76.9                     |
| 2                              |                                       |      | 0.562             | 0.417        |                          |
| 3                              |                                       |      | 0.527             | 0.436        |                          |
| Mean (± SD)                    |                                       |      | 0.540 ±0.019      | 0.415 ±0.022 |                          |
| OVERALL AVERAGE RECOVERY =     |                                       |      |                   |              | 75.0                     |

**TABLE 7: LONG TERM FREEZER STORAGE STABILITIES OF THE PRIMAQUINE HUMAN PLASMA ASSAY**

**Primaquine Concentration in Human Plasma Stored at -20°C**

| CONCENTRATION*        |      |     |     |      |
|-----------------------|------|-----|-----|------|
| (ng/ml)               |      |     |     |      |
| Spiked Concentration: | 85.4 | 171 | 456 | 1420 |
| TIME STORED           |      |     |     |      |
| 0 days                | 84.1 | 168 | 451 | 1450 |
| 1 day                 | 77.7 | 172 | 420 | 1380 |
| 2 days                | 85.2 | 153 | 424 | 1360 |
| 8 days                | 86.8 | 163 | 438 | 1340 |
| 2 weeks               | 79.9 | 162 | 424 | 1330 |
| 3 weeks               | 74.8 | 164 | 450 | 1440 |
| 1 month               | 86.2 | 163 | 434 | 1390 |
| 2 months              | 83.5 | 140 | 419 | 1180 |
| 3 months              | 88.6 | 154 | 412 | 1420 |

**Carboxy Metabolite Concentration in Human Plasma Stored at -20°C**

| CONCENTRATION*        |     |     |     |      |
|-----------------------|-----|-----|-----|------|
| (ng/ml)               |     |     |     |      |
| Spiked Concentration: | 150 | 300 | 800 | 2500 |
| TIME STORED           |     |     |     |      |
| 0 days                | 143 | 293 | 766 | 2450 |
| 1 day                 | 144 | 290 | 742 | 2370 |
| 2 days                | 135 | 255 | 741 | 2060 |
| 8 days                | 149 | 298 | 769 | 2460 |
| 2 weeks               | 142 | 286 | 749 | 2280 |
| 3 weeks               | 140 | 294 | 778 | 2060 |
| 1 month               | 138 | 294 | 730 | 2400 |
| 2 months              | 152 | 245 | 739 | 2120 |
| 3 months              | 141 | 272 | 780 | 2520 |

\*Measured concentrations are averages of two analyses.

**TABLE 8: LONG TERM FREEZER STORAGE STABILITIES OF THE  
PRIMAQUINE HUMAN PLASMA ASSAY**

**Primaquine Concentration in Human Plasma Stored at -70°C**

|                       |  | CONCENTRATION* |     |     |      |
|-----------------------|--|----------------|-----|-----|------|
|                       |  | (ng/ml)        |     |     |      |
| Spiked Concentration: |  | 85.4           | 171 | 456 | 1420 |
| TIME STORED           |  |                |     |     |      |
| 0 days                |  | 85.1           | 166 | 430 | 1400 |
| 8 days                |  | 83.7           | 162 | 424 | 1410 |
| 13 days               |  | 87.9           | 168 | 436 | 1420 |
| 19 days               |  | 83.4           | 164 | 419 | 1460 |
| 2 months              |  | 91.2           | 176 | 496 | 1450 |
| 3 months              |  | 73.3           | 150 | 406 | 1370 |
| 6 months              |  | 78.0           | 153 | 388 | 1430 |

**Carboxy Metabolite Concentration in Human Plasma Stored at -70°C**

|                       |  | CONCENTRATION* |     |     |      |
|-----------------------|--|----------------|-----|-----|------|
|                       |  | (ng/ml)        |     |     |      |
| Spiked Concentration: |  | 150            | 300 | 800 | 2500 |
| TIME STORED           |  |                |     |     |      |
| 0 days                |  | 156            | 308 | 800 | 2610 |
| 8 days                |  | 157            | 321 | 823 | 2440 |
| 13 days               |  | 153            | 303 | 811 | 2480 |
| 19 days               |  | 136            | 293 | 780 | 2590 |
| 2 months              |  | 153            | 309 | 778 | 2650 |
| 3 months              |  | 148            | 312 | 805 | 2540 |
| 6 months              |  | 161            | 326 | 813 | 2940 |

\*Measured concentrations are averages of two analyses.

**TABLE 9: BENCHTOP STABILITIES OF THE PRIMAQUINE HUMAN PLASMA ASSAY**

**Primaquine Concentration in Human Plasma Stored at Room Temperature**

|                       |  | CONCENTRATION* |     |     |      |
|-----------------------|--|----------------|-----|-----|------|
|                       |  | (ng/ml)        |     |     |      |
| Spiked Concentration: |  | 85.4           | 171 | 456 | 1420 |
| TIME STORED           |  |                |     |     |      |
| 0 hour                |  | 98.6           | 173 | 433 | 1340 |
| 2 hours               |  | 95.8           | 176 | 436 | 1330 |
| 4 hours               |  | 98.0           | 201 | 480 | 1480 |
| 6 hours               |  | 92.5           | 173 | 459 | 1490 |

**Carboxy Metabolite Concentration in Human Plasma Stored at Room Temperature**

|                       |  | CONCENTRATION* |     |     |      |
|-----------------------|--|----------------|-----|-----|------|
|                       |  | (ng/ml)        |     |     |      |
| Spiked Concentration: |  | 150            | 300 | 800 | 2500 |
| TIME STORED           |  |                |     |     |      |
| 0 hour                |  | 147            | 292 | 752 | 2210 |
| 2 hours               |  | 151            | 302 | 713 | 2200 |
| 4 hours               |  | 152            | 302 | 725 | 2320 |
| 6 hours               |  | 158            | 304 | 762 | 2300 |

**TABLE 10: SYSTEM STABILITIES OF THE PRIMAQUINE HUMAN PLASMA ASSAY**

**Primaquine Concentration of Prepared Samples  
Stored at Less Than or Equal to 10°C**

|                       |  | CONCENTRATION*<br>(ng/ml) |     |     |      |
|-----------------------|--|---------------------------|-----|-----|------|
| Spiked Concentration: |  | 85.4                      | 171 | 456 | 1420 |
| TIME STORED           |  |                           |     |     |      |
| 0 hour                |  | 85.3                      | 167 | 481 | 1450 |
| 48 hours              |  | 91.2                      | 176 | 496 | 1450 |

**Carboxy Metabolite Concentration of Prepared Samples  
Stored at Less Than or Equal to 10°C**

|                       |  | CONCENTRATION*<br>(ng/ml) |     |     |      |
|-----------------------|--|---------------------------|-----|-----|------|
| Spiked Concentration: |  | 150                       | 300 | 800 | 2500 |
| TIME STORED           |  |                           |     |     |      |
| 0 hour                |  | 159                       | 299 | 880 | 2520 |
| 48 hours              |  | 160                       | 320 | 917 | 2550 |

**TABLE 11: EFFECT OF REPEATED FREEZE AND THAW CYCLES IN THE PRIMAQUINE HUMAN PLASMA ASSAY**

| Spiked<br>Concentration | Primaquine free base  |                       | Carboxylated metabolite |                       |
|-------------------------|-----------------------|-----------------------|-------------------------|-----------------------|
|                         | Low<br>Concentration* | High<br>Concentration | Low<br>Concentration*   | High<br>Concentration |
|                         | (171 ng/ml)           | (1420 ng/ml)          | (300 ng/ml)             | (2500 ng/ml)          |
| Cycle                   |                       |                       |                         |                       |
| 1                       | 180                   | 1330                  | 312                     | 2200                  |
| 2                       | 183                   | 1140                  | 304                     | 2000                  |
| 3                       | 177                   | 1250                  | 295                     | 2020                  |
| 4                       | 185                   | 1570                  | 278                     | 2720                  |
| 5                       | 173                   | 1350                  | 272                     | 2180                  |

\*Measured concentrations are averages of two analyses.

## LABORATORY METHODOLOGY FOR GENTAMICIN/PAROMOMYCIN HUMAN PLASMA ASSAY, STUDY REPORT 24

### A. INSTRUMENTS

1. Waters Intelligent Sample Processor Model 717 (Waters Associates, Milford, MA) or equivalent.
2. LC-600 Shimadzu Pump (Shimadzu Corp., Kyoto, Japan) or equivalent. Two are required, one for mobile phase and one for post-column reagent.
3. Shimadzu RF 535 Fluorescence Detector (Shimadzu Corp., Kyoto, Japan) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.
6. T-mixer, Valco (Altech, Berkeley, CA)

### B. REAGENTS

1. Gentamicin sulfate (WR 073633), bottle no. BM 18591 (WRAIR, Washington D.C.).
2. Paromomycin sulfate (WR 035928), bottle no. BM 17861 (WRAIR, Washington D.C.).
3. Sisomicin (Internal Standard) (Sigma Chemical Co., St. Louis, MO).
4. Boric acid (Sigma Chemical Co., St. Louis, MO).
5. *o*-Pthaldialdehyde (Sigma Chemical Co., St. Louis, MO).
6. 2-Mercaptoethanol (Sigma Chemical Co., St. Louis, MO).
7. Sodium sulfate (Sigma Chemical Co., St. Louis, MO).
8. Sodium octane sulfonate (Sigma Chemical Co., St. Louis, MO).
9. Potassium hydroxide (Fisher Scientific, Fair Lawn, NJ).
10. Glacial acetic acid (Fisher Scientific, Fair Lawn, NJ).

\*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

11. Perchloric acid (Fisher Scientific, Fair Lawn, NJ).
12. Methanol (Fisher Scientific, Fair Lawn, NJ).

### C. ASSAY CONDITIONS

#### 1. DETECTOR

##### Settings

Wavelengths: Excitation 340 nm; Emission-430 nm

Range: 16

Lamp: USHIO XENON, TYPE UXL-155-LCA(S-LC)

#### 2. COLUMN

Capcell C18 type SG 120Å, 5 µm particle size, 4.6 x 150 mm (Shiseido) or equivalent.

#### 3. MOBILE PHASE SOLVENT SYSTEM

16% CH<sub>3</sub>CN, 0.2 M Na<sub>2</sub>SO<sub>4</sub>, 0.02 M sodium octanesulfonate, 0.1% acetic acid

#### 4. POST COLUMN REAGENT (Dissolve *o*-phthalaldehyde in methanol. Add boric acid and potassium hydroxide to half the water, mix, add *o*-phthalaldehyde solution, mix, filter then add remaining water and mercaptoethanol. Store in 4°C refrigerator, use within 4 days and keep in an ice bucket with an ice pack when running the HPLC system.)

Water/methanol/mercaptoethanol (99.4/0.5/0.1) (v/v/v), 0.201 molal boric acid, 0.218 molal KOH and 0.0008% *o*-phthalaldehyde.

#### 5. FLOW RATE

mobile phase: 1.0 ml/min

post column reagent: 0.3 ml/min

#### 6. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and checked for deterioration by comparison to a newly made solution (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 6 months after the preparation date).

- a. WR 073633 (gentamicin sulfate) for precision expressed as the free base concentration.

Prep date: 2/21/95

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent      | Conc. (mg/ml) |
|----------------|-------------------------|----------------|----------------|--------------|---------------|
| Standard Curve | 15.56                   | 0.607          | 10.13          | mobile phase | 1.00          |
| Precision      | 15.56                   | 0.607          | 10.11          | mobile phase | 1.00          |

\*obtained from WRAIR

- b. WR 035928 (paromomycin sulfate) for precision expressed as the free base concentration.

Prep date: 2/21/95

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent      | Conc. (mg/ml) |
|----------------|-------------------------|----------------|----------------|--------------|---------------|
| Standard Curve | 16.69                   | 0.646          | 10.05          | mobile phase | 1.00          |
| Precision      | 16.67                   | 0.646          | 10.05          | mobile phase | 1.00          |

\*obtained from WRAIR

- c. Sisomicin (internal standard).

Prep date: 2/21/95

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent      | Conc. (mg/ml) |
|---------------|-------------------------|---------------|----------------|--------------|---------------|
| Internal std. | 11.50                   | 1             | 11.5           | mobile phase | 1.00          |

7. WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and discarded when stock solutions were discarded or by 6 months after the preparation date).

- a. High concentration combined gentamicin/paromomycin (as free bases) solutions.

| Solution Type  | Conc. Diluted (mg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent      | Conc. (µg/ml) |
|----------------|-----------------------|---------------------|----------------|--------------|---------------|
| Standard Curve | 1.00                  | 2.00 each           | 20             | mobile phase | 100           |
| Precision      | 1.00                  | 2.00 each           | 20             | mobile phase | 100           |

- b. Low concentration combined gentamicin/paromomycin (as free bases) solutions.

| Solution Type  | Conc. Diluted (mg/ml) | Volume Diluted (ml) | QS Volume (ml) | Solvent      | Conc. (µg/ml) |
|----------------|-----------------------|---------------------|----------------|--------------|---------------|
| Standard Curve | 100                   | 2.00                | 20             | mobile phase | 10.0          |
| Precision      | 100                   | 2.00                | 20             | mobile phase | 10.0          |

## c. Sisomicin - Internal standard.

| Solution Type | Conc.<br>Diluted<br>(mg/ml) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent      | Conc.<br>(mg/ml) |
|---------------|-----------------------------|---------------------------|----------------------|--------------|------------------|
| Internal std. | 1.00                        | 2.00                      | 90                   | mobile phase | 0.22             |

## 8. RETENTION TIMES (subject to change depending on temperature and column performance).

- a. Paromomycin - 7.0 min
- b. Gentamicin - 15.5 min
- c. Sisomicin (Internal Standard) - 10.0 min

## 9. BLANK PLASMA

Human plasma (CPD or CPDA-1 as anticoagulant) was obtained from the San Francisco Irwin Memorial Blood Bank.

## 10. INJECTION VOLUME

10  $\mu$ l

## 11. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves are calculated by weighted linear regression where weights =  $1/y$ .

## 12. LOWER LIMIT OF QUANTITATION OF METHOD (The lower limits of quantitation (LLOQ) of the assay of human plasma for gentamicin and paromomycin (as free bases) were based on the interday and intraday low point validation results, on standard curve calibrator results, and a minimum 2 to 1 signal to noise ratio.)

0.100  $\mu$ g/ml gentamicin (as free base) in human plasma

0.100  $\mu$ g/ml paromomycin (as free base) in human plasma

## 13. VOLUME MEASUREMENT

Plasma sample volumes were measured with a 200  $\mu$ l or a 1000  $\mu$ l Gilson Pipetman. Hamilton syringes were used to measure standard and control solution volumes.

## 14. WISP OPERATING TEMPERATURE

Room temperature.

#### 15. COLUMN HEATER

Operating temperature: 40°C

#### 16. REACTION COIL

Teflon knitted tube: 5 m, internal diameter; 0.3 mm

### D. SAMPLE STORAGE

All samples were kept frozen at -70°C before analysis and thawed for preparation and analysis, unless specified otherwise.

### E. SAMPLE PREPARATION

1. If frozen, vortex specimens for 20 seconds after sample thaws.
2. Pipet 0.2 ml of a plasma sample into a clean glass culture tube.
3. Spike standard curve samples as shown in Section G "Generation of Standard Curve Calibrators."
4. Add 20 µl of internal standard (0.022 mg/ml Sisomicin) solution.
5. Add 20 µl of perchloric acid. Vortex for 1 min.
6. Centrifuge 10 minutes at 3000 g.
7. Transfer supernatant to WISP vial and inject onto HPLC column.

### F. QUALITY CONTROL

1. Content and frequency of blanks

A blank plasma sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

2. PIPETTE CALIBRATION

See SOP 2C-1.2.

3. BALANCE CALIBRATION

See SOP 2C-2.1

## G. GENERATION OF STANDARD CURVE CALIBRATORS

A representative example of the generation of standard curve calibrators is shown in the table below. Spike blank plasma standard curve samples with mixed gentamicin/paromomycin (as free bases) solution to make a standard curve. This procedure is equivalent to addition of the masses of gentamicin and paromomycin (as free bases) shown below. Since 0.200 ml plasma samples are assayed, these amounts correspond to the nominal free base concentrations shown below. Vortex for 10s.

Generation of Gentamicin/Paromomycin (as free base)  
Standard Curve Calibrators

| Sample | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Mass Spiked<br>(ng) | Standard Curve Sample Nominal Concentration<br>( $\mu$ g/ml) |
|--------|-----------------------------|-------------------------------------------------|---------------------|--------------------------------------------------------------|
| 00*    | 0                           | -                                               | 0                   | 0                                                            |
| 0**    | 0                           | -                                               | 0                   | 0                                                            |
| 1      | 2                           | 10                                              | 20                  | 0.100                                                        |
| 2      | 4                           | 10                                              | 40                  | 0.200                                                        |
| 3      | 8                           | 10                                              | 80                  | 0.400                                                        |
| 4      | 16                          | 10                                              | 160                 | 0.800                                                        |
| 5      | 3                           | 100                                             | 300                 | 1.50                                                         |
| 6      | 6                           | 100                                             | 600                 | 3.00                                                         |
| 7      | 12                          | 100                                             | 1200                | 6.00                                                         |
| 8      | 24                          | 100                                             | 2400                | 12.0                                                         |

## H. GENERATION OF PRECISION SAMPLES

Precision samples were generated by spiking 0.2 ml plasma specimens with control working solutions to make the gentamicin/paromomycin (as free bases) concentrations shown.

Generation of Gentamicin/Paromomycin (as free bases)  
Precision Samples

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution Concentration<br>( $\mu$ g/ml) | Control Volume<br>(ml) | Precision Sample Nominal Concentration<br>( $\mu$ g/ml) |
|------|-----------------------------|-------------------------------------------------|------------------------|---------------------------------------------------------|
| X-Lo | 4                           | 10.0                                            | 0.2                    | 0.200                                                   |
| Low  | 16                          | 10.0                                            | 0.2                    | 0.800                                                   |
| Med. | 5                           | 100                                             | 0.2                    | 2.50                                                    |
| Hi   | 10                          | 100                                             | 0.2                    | 5.00                                                    |

\*00 = Sample with no drug and no internal standard.

\*0 = Sample with no drug but with internal standard.

## I. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the gentamicin/paromomycin to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.2 ml) samples were spiked with gentamicin/paromomycin (and vortexed) then prepared as described above in "Sample Preparation," except no standard curve is used. Reference samples were generated by spiking 0.2 ml mobile phase with gentamicin/paromomycin then preparing the sample as described above in "Sample Preparation," except no standard curve is used.

## J. GENERATION OF STABILITY SAMPLES

1. Long term stability samples were generated by spiking pooled human plasma samples as shown below.

|      | Volume Spiked (μl) | Gentamicin/Paromomycin Free Base Spiking Solution Concentration (μg/ml) | Plasma Volume (ml) | Gentamicin Free Base Concentration (μg/ml) | Paromomycin Free Base Concentration (μg/ml) |
|------|--------------------|-------------------------------------------------------------------------|--------------------|--------------------------------------------|---------------------------------------------|
| X-Lo | 60                 | 100                                                                     | 14.940             | 0.400                                      | 0.400                                       |
| Low  | 150                | 100                                                                     | 14.850             | 1.00                                       | 1.00                                        |
| Med. | 375                | 100                                                                     | 14.625             | 2.50                                       | 2.50                                        |
| Hi   | 75                 | 1000                                                                    | 14.925             | 5.00                                       | 5.00                                        |

2. Benchtop stability samples were generated by spiking pooled human plasma samples as shown below.

|      | Volume Spiked (μl) | Gentamicin Free Base Spiking Solution Concentration (μg/ml) | Plasma Volume (ml) | Gentamicin Free Base Concentration (μg/ml) | Paromomycin Free Base Concentration (μg/ml) |
|------|--------------------|-------------------------------------------------------------|--------------------|--------------------------------------------|---------------------------------------------|
| X-Lo | 60                 | 100                                                         | 2.940              | 0.200                                      | 0.200                                       |
| Low  | 24                 | 100                                                         | 2.976              | 0.800                                      | 0.800                                       |
| Med. | 75                 | 100                                                         | 2.925              | 2.50                                       | 2.50                                        |
| Hi   | 150                | 100                                                         | 2.850              | 5.00                                       | 5.00                                        |

3. System stability samples were generated by spiking 0.2 ml human plasma specimens with gentamicin/paromomycin (as free bases) working solution as shown in Section H, "Generation of Precision Samples."
4. The effect of repeated freeze and thaw cycles on stability of gentamicin/paromomycin in human plasma samples was determined as described in laboratory SOP 2D-3.1, Section G.2. Freeze/thaw samples at Hi and Low concentrations were generated as described above for benchtop stability samples and in laboratory SOP 2D-3.1, Section G.2.

## K. VALIDATION RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in a representative standard curve for gentamicin/paromomycin appear in Figure 3. Peak height ratios for these calibrators appear in Table 1. Statistical parameters of plasma interday precision standard curve calibrators appear in Table 2.

### 2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Table 3.

### 3. LLOQ

Results for this evaluation appear in Table 4.

### 4. RECOVERY

Results for this evaluation appear in Table 5.

### 5. STABILITY

a. System Stability: Results appear in Table 6.

b. Long Term Stability: Results appear in Table 7.

c. Bench Top Stability: Results appear in Table 8.

d. Freeze/Thaw Stability: Results appear in Table 9.

### 6. BLIND SAMPLE ANALYSIS

Results appear in Table 10.

### 7. RAT PLASMA SHORT VALIDATION

Results appear in Tables 11-14.

TABLE 1A: REPRESENTATIVE STANDARD CURVE FOR  
GENTAMICIN HUMAN PLASMA ASSAY,  
STUDY REPORT 24

| SPIKED OR<br>DILUTION<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE<br>CONCENTRATION<br>(µg/ml) | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(µg/ml) |
|------------------------------------------|-----------------------------------------------|---------------------------|----------------------------------------|
| 0                                        | 0                                             | -                         | -                                      |
| 20                                       | 0.100                                         | 0.066                     | 0.0987                                 |
| 40                                       | 0.200                                         | 0.113                     | 0.211                                  |
| 80                                       | 0.400                                         | 0.195                     | 0.406                                  |
| 160                                      | 0.800                                         | 0.347                     | 0.768                                  |
| 300                                      | 1.50                                          | 0.634                     | 1.45                                   |
| 600                                      | 3.00                                          | 1.309                     | 3.06                                   |
| 1200                                     | 6.00                                          | 2.569                     | 6.06                                   |
| 2400                                     | 12.0                                          | 5.037                     | 11.9                                   |

Regression equation:\*\*\*

$$y = 0.420x + 0.0246, r^2 = 0.9997$$

\* In 0.2 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

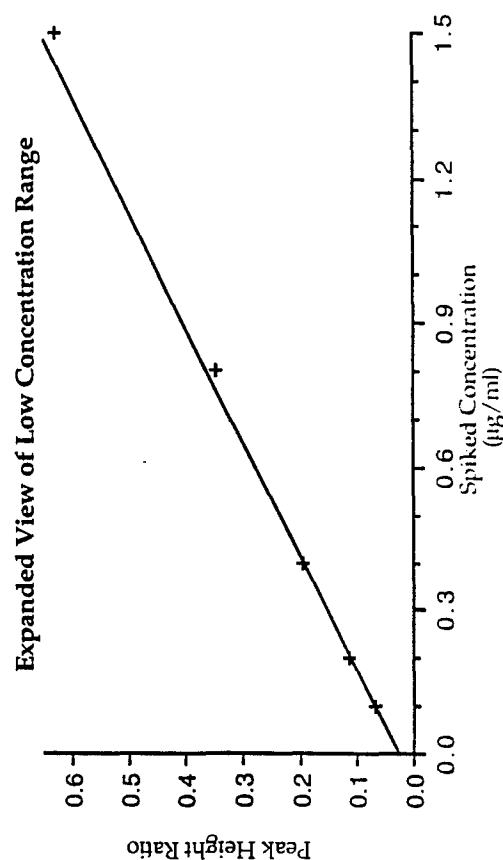
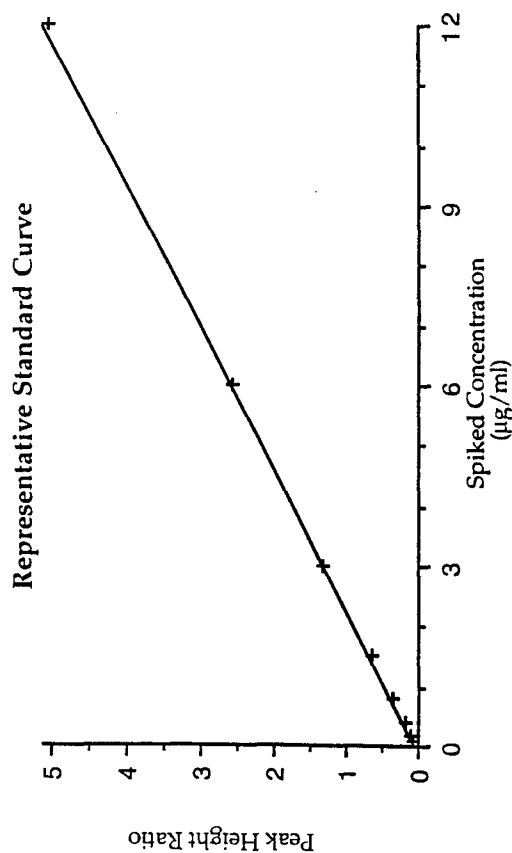


TABLE 1B: REPRESENTATIVE STANDARD CURVE FOR  
PAROMOMYCIN HUMAN PLASMA ASSAY,  
STUDY REPORT 24

| SPIKED OR<br>DILUTION<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE<br>CONCENTRATION<br>(µg/ml) | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(µg/ml) |
|------------------------------------------|-----------------------------------------------|---------------------------|----------------------------------------|
| 0                                        | 0                                             | -                         | -                                      |
| 20                                       | 0.100                                         | 0.181                     | 0.957                                  |
| 40                                       | 0.200                                         | 0.346                     | 0.201                                  |
| 80                                       | 0.400                                         | 0.665                     | 0.405                                  |
| 160                                      | 0.800                                         | 1.275                     | 0.794                                  |
| 300                                      | 1.50                                          | 2.347                     | 1.48                                   |
| 600                                      | 3.00                                          | 4.951                     | 3.14                                   |
| 1200                                     | 6.00                                          | 9.597                     | 6.10                                   |
| 2400                                     | 12.0                                          | 18.515                    | 11.8                                   |

Regression equation:\*\*\*

$$y = 1.57x + 0.0310, r^2 = 0.9994$$

\* In 0.2 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight = 1/y.

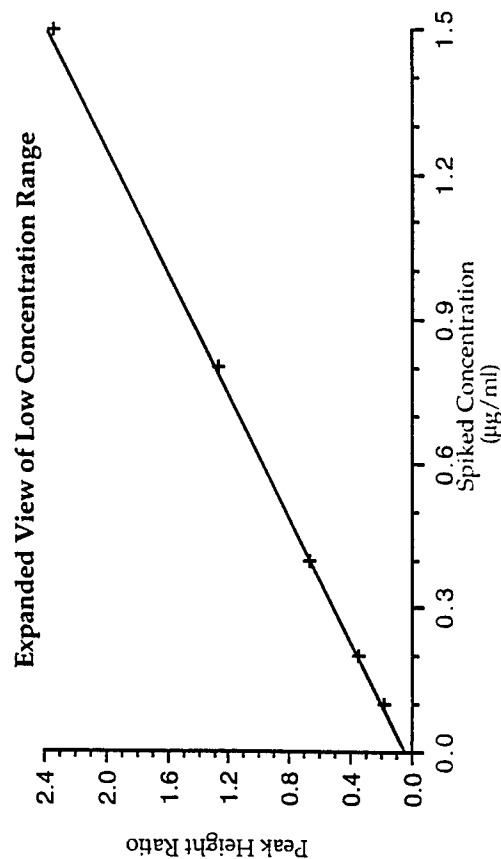
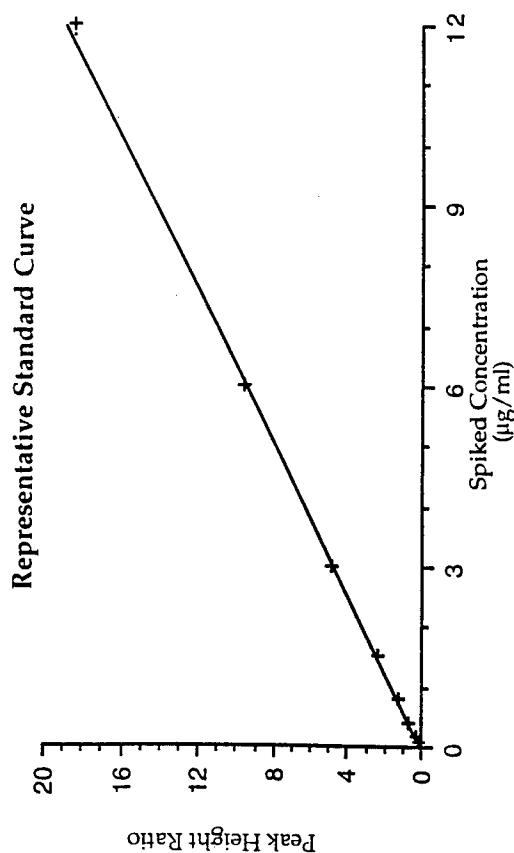


**TABLE 2A: PRECISION STANDARD CURVE DATA FOR GENTAMICIN  
HUMAN PLASMA ASSAY, STUDY REPORT 24**

Gentamicin Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|----------------|------------|------------|---------------------------------|
| 2/23/95                | interge1       | 0.46760957 | -0.0048544 | 0.99874855                      |
| 2/28/95                | intrage        | 0.4503625  | 0.00545335 | 0.99982051                      |
| 3/2/95                 | day1gent       | 0.43440038 | 0.00910677 | 0.99948823                      |
| 3/3/95                 | day2gent       | 0.43067307 | 0.00340248 | 0.9990281                       |
| 3/4/95                 | day3gent       | 0.41962867 | 0.02458418 | 0.99973472                      |
| 3/4/95                 | inter3ge       | 0.42586435 | -0.0015976 | 0.99909062                      |

Gentamicin Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |        |        |        |        |        |       |       |
|-------------------|---------------------------------------|--------|--------|--------|--------|--------|-------|-------|
|                   | 0.100                                 | 0.200  | 0.400  | 0.800  | 1.50   | 3.00   | 6.00  | 12.0  |
|                   | Back Calculated Concentration (µg/ml) |        |        |        |        |        |       |       |
| interge1          | 0.113                                 | 0.184  | 0.391  | 0.740  | 1.53   | 3.14   | 6.18  | 11.7  |
| intrage           | 0.0967                                | 0.197  | 0.399  | 0.852  | 1.49   | 3.01   | 5.96  | 12.0  |
| day1gent          | 0.119                                 | 0.184  | 0.382  | 0.787  | 1.46   | 3.06   | 6.12  | 11.9  |
| day2gent          | 0.106                                 | 0.206  | 0.410  | 0.772  | 1.47   | 2.85   | 5.89  | 12.3  |
| day3gent          | 0.0987                                | 0.211  | 0.406  | 0.768  | 1.45   | 3.06   | 6.06  | 11.9  |
| inter3ge          | 0.107                                 | 0.203  | 0.408  | 0.772  | 1.43   | 2.84   | 6.17  | 12.1  |
| n                 | 6                                     | 6      | 6      | 6      | 6      | 6      | 6     | 6     |
| Mean              | 0.107                                 | 0.198  | 0.399  | 0.782  | 1.47   | 2.99   | 6.06  | 12.0  |
| SD                | 0.00843                               | 0.0114 | 0.0110 | 0.0376 | 0.0349 | 0.122  | 0.117 | 0.204 |
| Percent CV        | 7.88                                  | 5.76   | 2.76   | 4.81   | 2.37   | 4.08   | 1.93  | 1.70  |
| Percent RE        | +7.00                                 | -1.00  | -0.250 | -2.25  | -2.00  | -0.333 | +1.00 | 0     |

**TABLE 2B: PRECISION STANDARD CURVE DATA FOR PAROMOMYCIN  
HUMAN PLASMA ASSAY, STUDY REPORT 24**

Paromomycin Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|----------------|------------|------------|---------------------------------|
| 2/23/95                | inter-1        | 1.98288996 | 0.04673078 | 0.99970962                      |
| 2/28/95                | intrapar       | 1.70096037 | 0.03214057 | 0.99932455                      |
| 3/2/95                 | day1pa         | 1.46449057 | -0.0329994 | 0.99893475                      |
| 3/3/95                 | day2par        | 1.36286881 | -0.0583854 | 0.99897777                      |
| 3/4/95                 | day3par        | 1.56709206 | 0.03098221 | 0.99941396                      |
| 3/4/95                 | inter3pa       | 1.34555578 | -0.0674372 | 0.99870615                      |

Paromomycin Back Calculated Standard Calibrators

| Validation<br>Run                     | Spiked Concentration (µg/ml) |        |        |        |        |       |        |        |
|---------------------------------------|------------------------------|--------|--------|--------|--------|-------|--------|--------|
|                                       | 0.100                        | 0.200  | 0.400  | 0.800  | 1.50   | 3.00  | 6.00   | 12.0   |
| Back Calculated Concentration (µg/ml) |                              |        |        |        |        |       |        |        |
| inter-1                               | 0.0990                       | 0.214  | 0.401  | 0.755  | 1.47   | 3.08  | 5.99   | 12.0   |
| intrapar                              | 0.0887                       | 0.196  | 0.401  | 0.867  | 1.55   | 3.10  | 5.99   | 11.8   |
| day1pa                                | 0.0881                       | bc     | 0.435  | 0.861  | 1.48   | 3.16  | 6.02   | 11.8   |
| day2par                               | 0.0883                       | 0.234  | 0.437  | 0.822  | 1.51   | 2.87  | 5.90   | 12.2   |
| day3par                               | 0.0957                       | 0.201  | 0.405  | 0.794  | 1.48   | 3.14  | 6.10   | 11.8   |
| inter3pa                              | 0.116                        | 0.192  | 0.365  | 0.770  | 1.45   | 2.88  | 6.26   | 12.0   |
| n                                     | 6                            | 5      | 6      | 6      | 6      | 6     | 6      | 6      |
| Mean                                  | 0.0960                       | 0.207  | 0.407  | 0.812  | 1.49   | 3.04  | 6.04   | 11.9   |
| SD                                    | 0.0108                       | 0.0170 | 0.0265 | 0.0466 | 0.0352 | 0.130 | 0.124  | 0.163  |
| Percent CV                            | 11.3                         | 8.21   | 6.51   | 5.74   | 2.36   | 4.28  | 2.05   | 1.37   |
| Percent RE                            | -4.00                        | +3.50  | +1.75  | +1.50  | -0.667 | +1.33 | +0.667 | -0.833 |

bc = unacceptable chromatogram.

TABLE 3A: PRECISION OF GENTAMICIN HUMAN PLASMA ASSAY

Interday Precision Gentamicin

| Validation<br>Run               | QC         | Spiked Concentrations (µg/mL) |        |       |        |
|---------------------------------|------------|-------------------------------|--------|-------|--------|
|                                 | Sample No. | 0.200                         | 0.800  | 2.50  | 5.00   |
| Measured Concentrations (µg/mL) |            |                               |        |       |        |
| interge1                        | 1          | 0.188                         | 0.808  | 2.50  | 5.07   |
|                                 | 2          | 0.222                         | 0.823  | 2.54  | 5.01   |
| intrage                         | 1          | 0.203                         | 0.796  | 2.52  | 4.87   |
|                                 | 2          | 0.199                         | 0.796  | 2.40  | 5.11   |
| day1gent                        | 1          | 0.189                         | 0.822  | 2.40  | 5.38   |
|                                 | 2          | 0.198                         | 0.833  | 2.37  | 5.12   |
| day2gent                        | 1          | 0.194                         | 0.756  | 2.41  | 4.84   |
|                                 | 2          | 0.192                         | 0.749  | 2.37  | 4.80   |
| day3gent                        | 1          | 0.218                         | 0.816  | 2.43  | 5.26   |
|                                 | 2          | 0.156                         | 0.802  | 2.56  | 5.12   |
| inter3ge                        | 1          | 0.194                         | 0.715  | 2.25  | 4.53   |
|                                 | 2          | 0.201                         | 0.743  | 2.23  | 4.35   |
| n                               |            | 12                            | 12     | 12    | 12     |
| Mean                            |            | 0.196                         | 0.788  | 2.42  | 4.96   |
| SD                              |            | 0.0165                        | 0.0379 | 0.105 | 0.295  |
| Percent CV                      |            | 8.42                          | 4.81   | 4.34  | 5.95   |
| Percent RE                      |            | -2.00                         | -1.50  | -3.20 | -0.800 |

Intraday Precision Gentamicin

| Validation                      | QC         | Spiked Concentrations (µg/mL) |        |        |       |
|---------------------------------|------------|-------------------------------|--------|--------|-------|
| Run                             | Sample No. | 0.200                         | 0.800  | 2.50   | 5.00  |
| Measured Concentrations (µg/mL) |            |                               |        |        |       |
| intra                           | 1          | 0.192                         | 0.789  | 2.27   | 4.95  |
|                                 | 2          | 0.183                         | 0.809  | 2.44   | 4.95  |
|                                 | 3          | 0.190                         | 0.803  | 2.38   | 4.73  |
|                                 | 4          | 0.190                         | 0.801  | 2.33   | 4.70  |
|                                 | 5          | 0.183                         | 0.792  | 2.36   | 4.74  |
|                                 | 6          | 0.177                         | 0.758  | 2.44   | 5.11  |
| n                               | 6          | 6                             | 6      | 6      |       |
| Mean                            |            | 0.186                         | 0.792  | 2.37   | 4.86  |
| SD                              |            | 0.00578                       | 0.0182 | 0.0657 | 0.165 |
| Percent CV                      |            | 3.11                          | 2.30   | 2.77   | 3.40  |
| Percent RE                      |            | -7.00                         | -1.00  | -5.20  | -2.80 |

TABLE 3B: PRECISION OF PAROMOMYCIN HUMAN PLASMA ASSAY

Interday Precision Paromomycin

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (µg/mL) |        |       |        |
|---------------------------------|------------------|-------------------------------|--------|-------|--------|
|                                 |                  | 0.200                         | 0.800  | 2.50  | 5.00   |
| Measured Concentrations (µg/mL) |                  |                               |        |       |        |
| inter-1                         | 1                | 0.184                         | 0.807  | 2.67  | 5.17   |
|                                 | 2                | 0.197                         | 0.837  | 2.58  | 5.05   |
| intrapar                        | 1                | 0.217                         | 0.895  | 2.78  | 5.40   |
|                                 | 2                | 0.195                         | 0.876  | 2.64  | 5.69   |
| day1par                         | 1                | 0.175                         | 0.862  | 2.23  | 5.21   |
|                                 | 2                | 0.186                         | 0.877  | 2.40  | 5.05   |
| day2par                         | 1                | 0.183                         | 0.769  | 2.36  | 4.70   |
|                                 | 2                | 0.181                         | 0.749  | 2.30  | 4.65   |
| day3par                         | 1                | 0.192                         | 0.846  | 2.53  | 5.41   |
|                                 | 2                | 0.202                         | 0.831  | 2.66  | 5.24   |
| inter3par                       | 1                | 0.186                         | 0.703  | 2.17  | 4.36   |
|                                 | 2                | 0.193                         | 0.734  | 2.14  | 4.18   |
| n                               |                  | 12                            | 12     | 12    | 12     |
| Mean                            |                  | 0.191                         | 0.816  | 2.46  | 5.01   |
| SD                              |                  | 0.0112                        | 0.0629 | 0.217 | 0.451  |
| Percent CV                      |                  | 5.86                          | 7.71   | 8.82  | 9.00   |
| Percent RE                      |                  | -4.50                         | +2.00  | -1.60 | +0.200 |

Intraday Precision Paromomycin

| Validation                      | QC         | Spiked Concentrations (µg/mL) |        |        |       |
|---------------------------------|------------|-------------------------------|--------|--------|-------|
| Run No.                         | Sample No. | 0.200                         | 0.800  | 2.50   | 5.00  |
| Measured Concentrations (µg/mL) |            |                               |        |        |       |
| intrapar                        | 1          | 0.188                         | 0.845  | 2.41   | 5.05  |
|                                 | 2          | 0.186                         | 0.837  | 2.45   | 5.13  |
|                                 | 3          | 0.189                         | 0.827  | 2.40   | 4.73  |
|                                 | 4          | 0.187                         | 0.810  | 2.33   | 4.73  |
|                                 | 5          | 0.184                         | 0.792  | 2.33   | 4.72  |
|                                 | 6          | 0.173                         | 0.781  | 2.21   | 5.05  |
| n                               |            | 6                             | 6      | 6      | 6     |
| Mean                            |            | 0.185                         | 0.815  | 2.36   | 4.90  |
| SD                              |            | 0.00589                       | 0.0254 | 0.0853 | 0.194 |
| Percent CV                      |            | 3.18                          | 3.12   | 3.61   | 3.96  |
| Percent RE                      |            | -7.50                         | +1.87  | -5.60  | -2.00 |

TABLE 4: LOWER LIMIT OF QUANTITATION OF THE HUMAN PLASMA  
ASSAY FOR GENTAMICIN/PAROMOMYCIN

## Gentamicin

| Spiked Concentration | 0.100 µg/ml             | 0.100 µg/ml |
|----------------------|-------------------------|-------------|
|                      | Measured Concentrations |             |
| Sample               | (µg/ml)                 |             |
|                      | Interday                | Intraday    |
| 1                    | 0.113                   | 0.111       |
| 2                    | 0.0967                  | 0.0799      |
| 3                    | 0.119                   | 0.0799      |
| 4                    | 0.106                   | 0.0732      |
| 5                    | 0.0987                  | 0.0799      |
| 6                    | 0.107                   | 0.0866      |
| Mean                 | 0.107                   | 0.0851      |
| SD                   | 0.00843                 | 0.0134      |
| Percent CV           | 7.88                    | 15.7        |
| Percent RE           | 7.00                    | -14.9       |

## Paromomycin

| Spiked Concentration | 0.100 µg/ml             | 0.100 µg/ml |
|----------------------|-------------------------|-------------|
|                      | Measured Concentrations |             |
| Sample               | (µg/ml)                 |             |
|                      | Interday                | Intraday    |
| 1                    | 0.0990                  | 0.101       |
| 2                    | 0.0887                  | 0.0851      |
| 3                    | 0.0881                  | 0.0877      |
| 4                    | 0.0883                  | 0.0741      |
| 5                    | 0.0957                  | 0.0870      |
| 6                    | 0.116                   | 0.0851      |
| Mean                 | 0.0960                  | 0.0867      |
| SD                   | 0.0108                  | 0.00860     |
| Percent CV           | 11.3                    | 9.92        |
| Percent RE           | -4.00                   | -13.3       |

TABLE 5: RECOVERY OF GENTAMICIN/PAROMOMYCIN FROM HUMAN PLASMA

| SAMPLE ID                      | SPIKED CONCENTRATION Range | (µg/ml) | PEAK HEIGHT RATIO |               | MEAN PERCENT RECOVERY |
|--------------------------------|----------------------------|---------|-------------------|---------------|-----------------------|
|                                |                            |         | SOLVENT           | PLASMA        |                       |
| Gentamicin                     |                            |         |                   |               |                       |
| 1                              | X Low                      | 0.200   | 0.080             | 0.081         | 89.0                  |
| 2                              |                            |         | 0.109             | 0.085         |                       |
| 3                              |                            |         | 0.111             | 0.100         |                       |
| Mean (± SD)                    |                            |         | 0.100 ± 0.017     | 0.089 ± 0.010 |                       |
| 1                              | Low                        | 0.800   | 0.349             | 0.383         | 102                   |
| 2                              |                            |         | 0.365             | 0.361         |                       |
| 3                              |                            |         | 0.382             | 0.379         |                       |
| Mean (± SD)                    |                            |         | 0.365 ± 0.017     | 0.374 ± 0.012 |                       |
| 1                              | Medium                     | 2.50    | 1.140             | 1.005         | 90.6                  |
| 2                              |                            |         | 1.085             | 1.069         |                       |
| 3                              |                            |         | 1.169             | 1.002         |                       |
| Mean (± SD)                    |                            |         | 1.131 ± 0.043     | 1.025 ± 0.038 |                       |
| 1                              | High                       | 5.00    | 2.225             | 2.079         | 94.2                  |
| 2                              |                            |         | 2.306             | 2.237         |                       |
| 3                              |                            |         | 2.277             | 2.097         |                       |
| Mean (± SD)                    |                            |         | 2.269 ± 0.041     | 2.138 ± 0.086 |                       |
| AVERAGE =                      |                            |         |                   |               | 94.0                  |
| Paromomycin                    |                            |         |                   |               |                       |
| 1                              | X Low                      | 0.200   | 0.360             | 0.393         | 91.3                  |
| 2                              |                            |         | 0.356             | 0.301         |                       |
| 3                              |                            |         | bc                | 0.288         |                       |
| Mean (± SD)                    |                            |         | 0.358 ± 0.003     | 0.327 ± 0.057 |                       |
| 1                              | Low                        | 0.800   | 1.330             | 1.416         | 102                   |
| 2                              |                            |         | 1.380             | 1.417         |                       |
| 3                              |                            |         | 1.420             | 1.362         |                       |
| Mean (± SD)                    |                            |         | 1.377 ± 0.045     | 1.398 ± 0.031 |                       |
| 1                              | Medium                     | 2.50    | 4.000             | 3.716         | 90.4                  |
| 2                              |                            |         | 3.990             | 3.922         |                       |
| 3                              |                            |         | 4.470             | 3.627         |                       |
| Mean (± SD)                    |                            |         | 4.153 ± 0.274     | 3.755 ± 0.151 |                       |
| 1                              | High                       | 5.00    | 8.290             | 7.399         | 91.5                  |
| 2                              |                            |         | 8.220             | 8.095         |                       |
| 3                              |                            |         | 8.620             | 7.501         |                       |
| Mean (± SD)                    |                            |         | 8.377 ± 0.214     | 7.665 ± 0.376 |                       |
| AVERAGE =                      |                            |         |                   |               | 93.8                  |
| bc = unacceptable chromatogram |                            |         |                   |               |                       |

TABLE 6A: SYSTEM STABILITY OF GENTAMICIN IN PREPARED SAMPLES

|                       |            | CONCENTRATION AT ROOM TEMPERATURE STORAGE ( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|----------------------------------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.200                                                          | 0.800 | 2.50  | 5.00  |
| TIME STORED           |            |                                                                |       |       |       |
| 0 days                | Sample 1   | 0.192                                                          | 0.826 | 2.62  | 5.26  |
|                       | Sample 2   | 0.204                                                          | 0.798 | 2.65  | 5.26  |
|                       | Mean       | 0.198                                                          | 0.812 | 2.64  | 5.26  |
|                       | Percent RE | -1.00                                                          | +1.50 | +5.40 | +5.20 |
| 1 day                 | Sample 1   | 0.215                                                          | 0.849 | 2.70  | 5.34  |
|                       | Sample 2   | 0.232                                                          | 0.804 | 2.67  | 5.26  |
|                       | Mean       | 0.224                                                          | 0.827 | 2.69  | 5.30  |
|                       | Percent RE | +11.8                                                          | +3.31 | +7.40 | +6.00 |
| 2 days                | Sample 1   | 0.217                                                          | 0.849 | 2.69  | 5.19  |
|                       | Sample 2   | 0.202                                                          | 0.858 | 2.69  | 5.20  |
|                       | Mean       | 0.210                                                          | 0.854 | 2.69  | 5.20  |
|                       | Percent RE | +4.75                                                          | +6.69 | +7.60 | +3.90 |
| 3 days                | Sample 1   | bc                                                             | 0.896 | 2.71  | 5.43  |
|                       | Sample 2   | 0.215                                                          | 0.868 | 2.70  | 5.18  |
|                       | Mean       | 0.215                                                          | 0.882 | 2.71  | 5.31  |
|                       | Percent RE | +7.50                                                          | +10.3 | +8.20 | +6.10 |
| 5 days                | Sample 1   | 0.219                                                          | 0.887 | 2.73  | 5.32  |
|                       | Sample 2   | 0.219                                                          | 0.885 | 2.75  | 5.31  |
|                       | Mean       | 0.219                                                          | 0.886 | 2.74  | 5.32  |
|                       | Percent RE | +9.50                                                          | +10.8 | +9.60 | +6.30 |
| 6 days                | Sample 1   | 0.230                                                          | 0.909 | 2.80  | 5.42  |
|                       | Sample 2   | 0.232                                                          | 0.894 | 2.80  | ns    |
|                       | Mean       | 0.231                                                          | 0.902 | 2.80  | 5.42  |
|                       | Percent RE | +15.5                                                          | +12.7 | +12.0 | +8.40 |

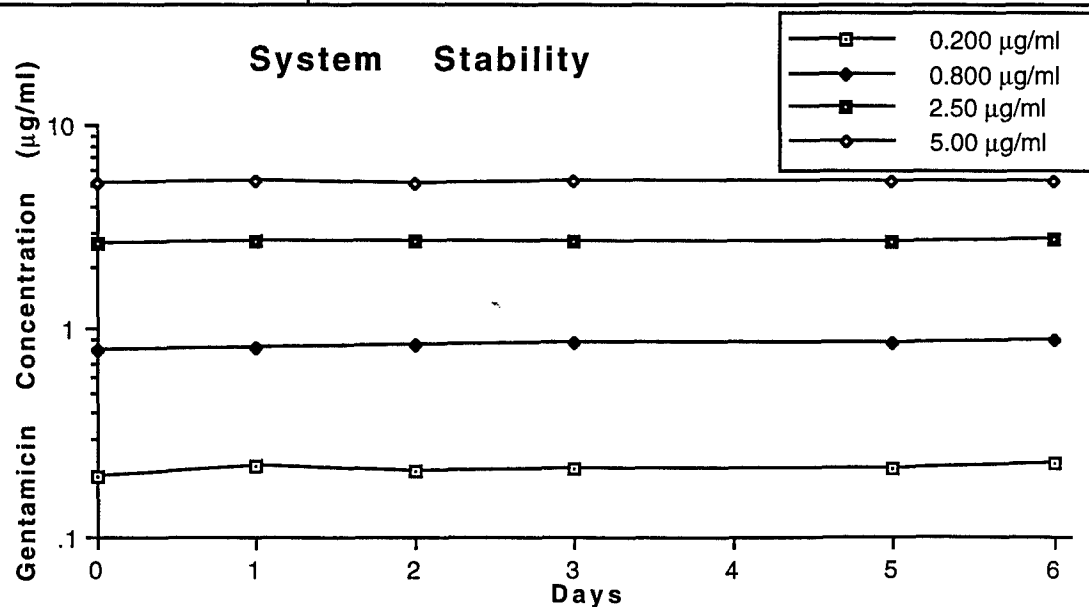


TABLE 6B: SYSTEM STABILITY OF PAROMOMYCIN IN PREPARED SAMPLES

|                       |            | CONCENTRATION AT ROOM TEMPERATURE STORAGE ( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|----------------------------------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.200                                                          | 0.800 | 2.50  | 5.00  |
| TIME STORED           |            |                                                                |       |       |       |
| 0 days                | Sample 1   | 0.208                                                          | 0.863 | 2.83  | 5.81  |
|                       | Sample 2   | 0.202                                                          | 0.791 | 2.55  | 5.64  |
|                       | Mean       | 0.205                                                          | 0.827 | 2.69  | 5.73  |
|                       | Percent RE | +2.50                                                          | +3.37 | +7.60 | +14.5 |
| 1 day                 | Sample 1   | 0.220                                                          | 0.919 | 2.98  | 5.83  |
|                       | Sample 2   | 0.204                                                          | 0.834 | 2.70  | 5.74  |
|                       | Mean       | 0.212                                                          | 0.877 | 2.84  | 5.79  |
|                       | Percent RE | +6.00                                                          | +9.56 | +13.6 | +15.7 |
| 2 days                | Sample 1   | 0.232                                                          | 0.922 | 2.82  | 5.29  |
|                       | Sample 2   | 0.219                                                          | 0.861 | 2.56  | 5.35  |
|                       | Mean       | 0.226                                                          | 0.892 | 2.69  | 5.32  |
|                       | Percent RE | +12.8                                                          | +11.4 | +7.60 | +6.40 |
| 3 days                | Sample 1   | 0.209                                                          | 0.824 | 2.64  | 5.23  |
|                       | Sample 2   | 0.192                                                          | 0.798 | 2.47  | 5.12  |
|                       | Mean       | 0.201                                                          | 0.811 | 2.56  | 5.18  |
|                       | Percent RE | 0.250                                                          | +1.37 | +2.20 | +3.50 |
| 5 days                | Sample 1   | 0.173                                                          | 0.773 | 2.47  | 4.96  |
|                       | Sample 2   | 0.183                                                          | 0.739 | 2.36  | 4.84  |
|                       | Mean       | 0.178                                                          | 0.756 | 2.42  | 4.90  |
|                       | Percent RE | -11.0                                                          | -5.50 | -3.40 | -2.00 |
| 6 days                | Sample 1   | 0.162                                                          | 0.691 | 2.16  | 4.23  |
|                       | Sample 2   | 0.169                                                          | 0.683 | 2.16  | rs    |
|                       | Mean       | 0.166                                                          | 0.687 | 2.16  | 4.23  |
|                       | Percent RE | -17.3                                                          | -14.1 | -13.6 | -15.4 |

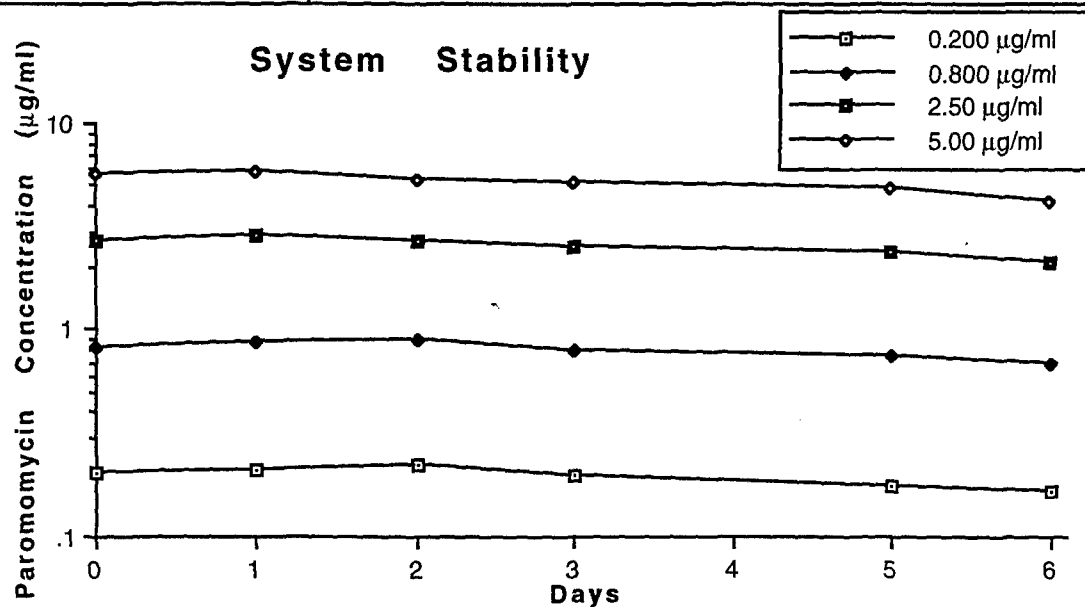


**TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA**

**GENTAMICIN CONCENTRATION IN PLASMA STORED AT -20°C**

| Gentamicin            |            | CONCENTRATION<br>( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|---------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                                 | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                                       |       |       |       |
| 0 days                | Sample 1   | 0.299                                 | 0.978 | 2.29  | 5.11  |
|                       | Sample 2   | 0.332                                 | 0.933 | 2.48  | 4.95  |
|                       | Mean       | 0.316                                 | 0.956 | 2.39  | 5.03  |
|                       | Percent RE | -21.1                                 | -4.45 | -4.60 | +0.60 |
| 1 days                | Sample 1   | 0.400                                 | 1.00  | 2.46  | 4.89  |
|                       | Sample 2   | 0.327                                 | 0.902 | 2.37  | 4.78  |
|                       | Mean       | 0.364                                 | 0.951 | 2.42  | 4.84  |
|                       | Percent RE | -9.12                                 | -4.90 | -3.40 | -3.30 |
| 2 days                | Sample 1   | 0.340                                 | 0.881 | 2.29  | 4.77  |
|                       | Sample 2   | 0.313                                 | 0.905 | 2.25  | 4.60  |
|                       | Mean       | 0.327                                 | 0.893 | 2.27  | 4.69  |
|                       | Percent RE | -18.4                                 | -10.7 | -9.20 | -6.30 |
| 3 days                | Sample 1   | 0.325                                 | 0.880 | 2.29  | 4.80  |
|                       | Sample 2   | 0.306                                 | 0.890 | 2.31  | 3.98  |
|                       | Mean       | 0.316                                 | 0.885 | 2.30  | 4.39  |
|                       | Percent RE | -21.1                                 | -11.5 | -8.00 | -12.2 |
| 1 week                | Sample 1   | 0.420                                 | 1.01  | 2.24  | 4.92  |
|                       | Sample 2   | 0.325                                 | 0.972 | 2.35  | 4.67  |
|                       | Mean       | 0.373                                 | 0.991 | 2.30  | 4.80  |
|                       | Percent RE | -6.88                                 | -0.90 | -8.20 | -4.10 |
| 2 weeks               | Sample 1   | 0.299                                 | 0.878 | 2.30  | 4.89  |
|                       | Sample 2   | 0.297                                 | 0.916 | 2.39  | 4.88  |
|                       | Mean       | 0.298                                 | 0.897 | 2.35  | 4.89  |
|                       | Percent RE | -25.5                                 | -10.3 | -6.20 | -2.30 |
| 3 weeks               | Sample 1   | 0.276                                 | 0.825 | 2.31  | 4.80  |
|                       | Sample 2   | 0.285                                 | 0.816 | 2.13  | 4.52  |
|                       | Mean       | 0.281                                 | 0.821 | 2.22  | 4.66  |
|                       | Percent RE | -29.9                                 | -18.0 | -11.2 | -6.80 |
| 1 month               | Sample 1   | 0.316                                 | 0.896 | 2.23  | 4.63  |
|                       | Sample 2   | 0.280                                 | 0.915 | 2.26  | 4.46  |
|                       | Mean       | 0.298                                 | 0.906 | 2.25  | 4.55  |
|                       | Percent RE | -25.5                                 | -9.45 | -10.2 | -9.10 |

**TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA**

**GENTAMICIN CONCENTRATION IN PLASMA STORED AT -20°C**

| Gentamicin            |            | CONCENTRATION<br>( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|---------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                                 | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                                       |       |       |       |
| 2 months              | Sample 1   | 0.320                                 | 0.872 | 2.30  | 4.01  |
|                       | Sample 2   | 0.328                                 | 0.883 | 2.08  | 4.06  |
|                       | Mean       | 0.324                                 | 0.878 | 2.19  | 4.04  |
|                       | Percent RE | -19.0                                 | -12.3 | -12.4 | -19.3 |
| 3 months              | Sample 1   | 0.269                                 | 0.781 | 1.98  | 3.92  |
|                       | Sample 2   | 0.288                                 | 0.781 | 1.96  | 3.91  |
|                       | Mean       | 0.279                                 | 0.781 | 1.97  | 3.92  |
|                       | Percent RE | -30.4                                 | -21.9 | -21.2 | -21.7 |
| 6 months              | Sample 1   | 0.291                                 | 0.625 | 1.72  | 3.10  |
|                       | Sample 2   | 0.280                                 | 0.701 | 1.70  | 3.15  |
|                       | Mean       | 0.286                                 | 0.663 | 1.71  | 3.13  |
|                       | Percent RE | -28.6                                 | -33.7 | -31.6 | -37.5 |
| 1 year                | Sample 1   | 0.272                                 | 0.560 | 1.38  | 2.68  |
|                       | Sample 2   | 0.213                                 | 0.664 | 1.60  | 2.85  |
|                       | Mean       | 0.243                                 | 0.612 | 1.49  | 2.77  |
|                       | Percent RE | -39.4                                 | -38.8 | -40.4 | -44.7 |

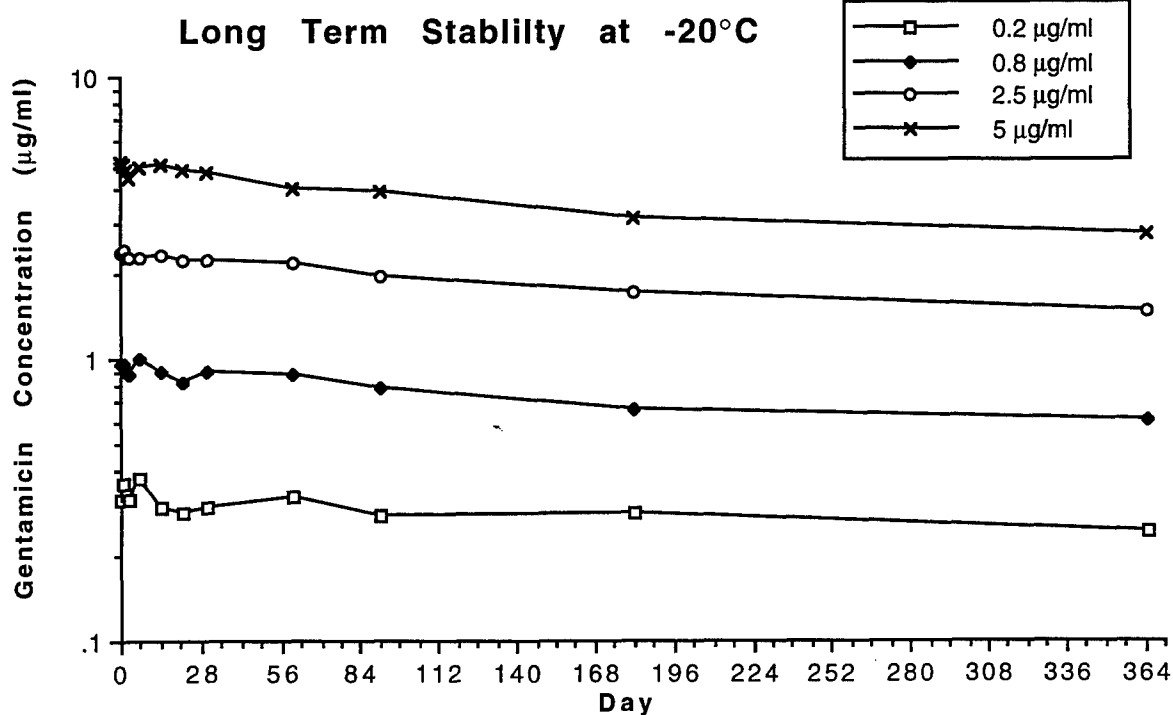


**TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA**

**GENTAMICIN CONCENTRATION IN PLASMA STORED AT -70°C**

|                       |            | CONCENTRATION<br>(µg/ml) |       |       |       |
|-----------------------|------------|--------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                    | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                          |       |       |       |
| 0 days                | Sample 1   | 0.328                    | 0.949 | 2.30  | 4.87  |
|                       | Sample 2   | 0.323                    | 0.940 | 2.40  | 4.96  |
|                       | Mean       | 0.326                    | 0.945 | 2.35  | 4.92  |
|                       | Percent RE | -18.6                    | -5.55 | -6.00 | -1.70 |
| 1 day                 | Sample 1   | 0.345                    | 0.895 | 2.33  | 4.91  |
|                       | Sample 2   | 0.389                    | 0.990 | 2.41  | 4.93  |
|                       | Mean       | 0.367                    | 0.943 | 2.37  | 4.92  |
|                       | Percent RE | -8.25                    | -5.75 | -5.20 | -1.60 |
| 2 days                | Sample 1   | 0.315                    | 0.900 | 2.32  | 4.75  |
|                       | Sample 2   | 0.343                    | 0.867 | 2.29  | 4.83  |
|                       | Mean       | 0.329                    | 0.884 | 2.31  | 4.79  |
|                       | Percent RE | -17.8                    | -11.7 | -7.80 | -4.20 |
| 3 days                | Sample 1   | 0.246                    | 0.845 | 2.32  | 4.78  |
|                       | Sample 2   | 0.294                    | 0.871 | 2.33  | 4.82  |
|                       | Mean       | 0.270                    | 0.858 | 2.33  | 4.80  |
|                       | Percent RE | -32.5                    | -14.2 | -7.00 | -4.00 |
| 1 week                | Sample 1   | 0.330                    | 0.967 | 2.52  | 5.09  |
|                       | Sample 2   | 0.310                    | 0.925 | 2.41  | 4.74  |
|                       | Mean       | 0.320                    | 0.946 | 2.47  | 4.92  |
|                       | Percent RE | -20.0                    | -5.40 | -1.40 | -1.70 |
| 2 weeks               | Sample 1   | 0.400                    | 0.916 | 2.32  | 4.86  |
|                       | Sample 2   | 0.411                    | 0.943 | 2.31  | 4.83  |
|                       | Mean       | 0.406                    | 0.930 | 2.32  | 4.85  |
|                       | Percent RE | +1.37                    | -7.05 | -7.40 | -3.10 |
| 3 weeks               | Sample 1   | 0.316                    | 0.878 | 2.28  | 4.38  |
|                       | Sample 2   | 0.311                    | 0.934 | 2.24  | 4.57  |
|                       | Mean       | 0.314                    | 0.906 | 2.26  | 4.48  |
|                       | Percent RE | -21.6                    | -9.40 | -9.60 | -10.5 |
| 1 month               | Sample 1   | 0.310                    | 0.862 | 2.25  | 4.68  |
|                       | Sample 2   | 0.320                    | 0.862 | 2.25  | 4.68  |
|                       | Mean       | 0.315                    | 0.862 | 2.25  | 4.68  |
|                       | Percent RE | -21.3                    | -13.8 | -10.0 | -6.40 |

**TABLE 7A: LONG TERM STABILITY OF GENTAMICIN IN HUMAN PLASMA**

GENTAMICIN CONCENTRATION IN PLASMA STORED AT -70°C

|                       |            | CONCENTRATION<br>( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|---------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                                 | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                                       |       |       |       |
| 2 months              | Sample 1   | 0.298                                 | 0.861 | 2.69  | 4.79  |
|                       | Sample 2   | 0.306                                 | 0.835 | 2.24  | 4.55  |
|                       | Mean       | 0.302                                 | 0.848 | 2.47  | 4.67  |
|                       | Percent RE | -24.5                                 | -15.2 | -1.40 | -6.60 |
| 3 months              | Sample 1   | 0.315                                 | 0.892 | 2.35  | 4.91  |
|                       | Sample 2   | 0.317                                 | 0.914 | 2.36  | 4.82  |
|                       | Mean       | 0.316                                 | 0.903 | 2.36  | 4.87  |
|                       | Percent RE | -21.0                                 | -9.70 | -5.80 | -2.70 |
| 6 months              | Sample 1   | 0.412                                 | 1.05  | 2.45  | 4.89  |
|                       | Sample 2   | 0.384                                 | 1.03  | 2.47  | 4.85  |
|                       | Mean       | 0.398                                 | 1.04  | 2.46  | 4.87  |
|                       | Percent RE | -0.50                                 | +4.00 | -1.60 | -2.60 |
| 1 year                | Sample 1   | 0.356                                 | 0.959 | 2.25  | 4.59  |
|                       | Sample 2   | 0.331                                 | 0.902 | 2.31  | 4.68  |
|                       | Mean       | 0.344                                 | 0.931 | 2.28  | 4.64  |
|                       | Percent RE | -14.1                                 | -6.95 | -8.80 | -7.30 |

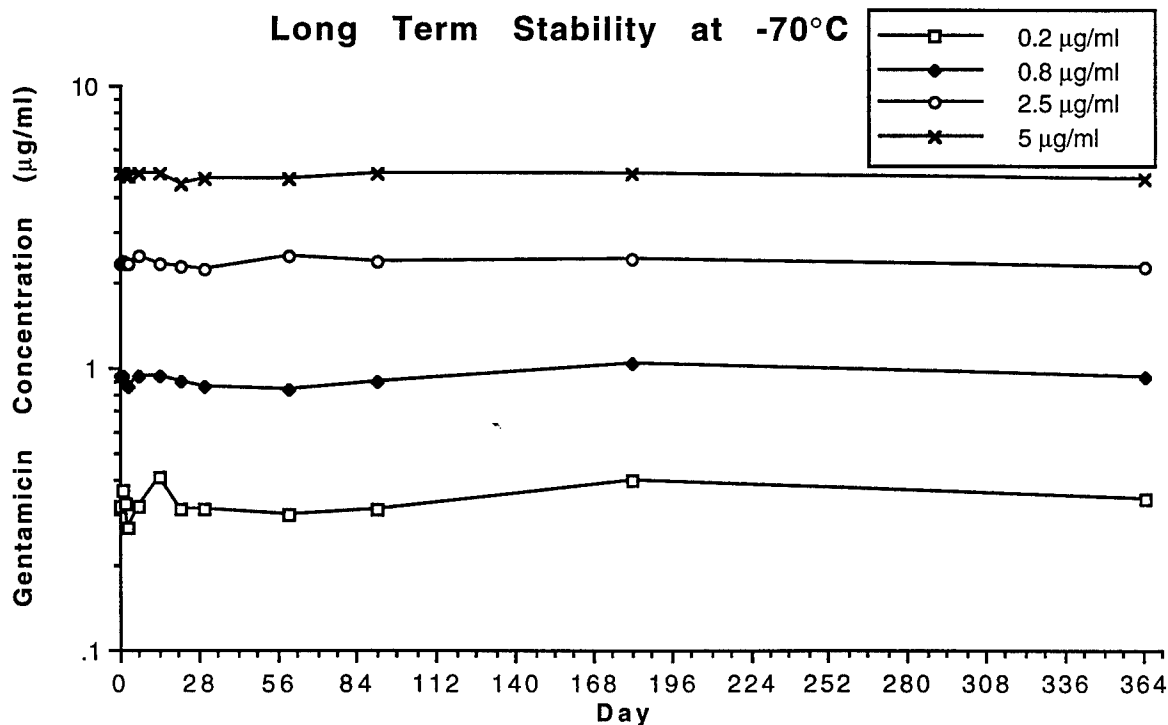


**TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA**

**PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -20°C**

| Paromomycin           |            | CONCENTRATION<br>(µg/ml) |       |       |       |
|-----------------------|------------|--------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                    | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                          |       |       |       |
| 0 days                | Sample 1   | 0.365                    | 1.07  | 2.41  | 5.16  |
|                       | Sample 2   | 0.456                    | 1.16  | 2.85  | 5.40  |
|                       | Mean       | 0.411                    | 1.12  | 2.63  | 5.28  |
|                       | Percent RE | +2.62                    | +11.5 | +5.20 | +5.60 |
| 1 day                 | Sample 1   | 0.457                    | 1.20  | 2.95  | 5.70  |
|                       | Sample 2   | 0.410                    | 1.03  | 2.60  | 5.00  |
|                       | Mean       | 0.434                    | 1.12  | 2.78  | 5.35  |
|                       | Percent RE | +8.37                    | +11.5 | +11.0 | +7.00 |
| 2 days                | Sample 1   | 0.438                    | 1.09  | 2.77  | 5.67  |
|                       | Sample 2   | 0.377                    | 0.992 | 2.39  | 4.77  |
|                       | Mean       | 0.408                    | 1.04  | 2.58  | 5.22  |
|                       | Percent RE | +1.87                    | +4.10 | +3.20 | +4.40 |
| 3 days                | Sample 1   | 0.389                    | 1.04  | 2.55  | 5.17  |
|                       | Sample 2   | 0.385                    | 1.02  | 2.51  | 4.23  |
|                       | Mean       | 0.387                    | 1.03  | 2.53  | 4.70  |
|                       | Percent RE | -3.25                    | +3.00 | +1.20 | -6.00 |
| 1 week                | Sample 1   | 0.430                    | 1.21  | 3.08  | 6.67  |
|                       | Sample 2   | 0.440                    | 1.17  | 3.24  | 6.34  |
|                       | Mean       | 0.435                    | 1.19  | 3.16  | 6.51  |
|                       | Percent RE | +8.75                    | +19.0 | +26.4 | +30.1 |
| 2 weeks               | Sample 1   | 0.415                    | 1.15  | 2.54  | 5.58  |
|                       | Sample 2   | 0.411                    | 1.09  | 2.73  | 5.62  |
|                       | Mean       | 0.413                    | 1.12  | 2.64  | 5.60  |
|                       | Percent RE | +3.25                    | +12.0 | +5.40 | +12.0 |
| 3 weeks               | Sample 1   | 0.304                    | 0.763 | 2.22  | 3.68  |
|                       | Sample 2   | 0.326                    | 0.782 | 1.85  | 4.10  |
|                       | Mean       | 0.315                    | 0.773 | 2.04  | 3.89  |
|                       | Percent RE | -21.3                    | -22.8 | -18.6 | -22.2 |
| 1 month               | Sample 1   | 0.388                    | 1.07  | 2.54  | 5.15  |
|                       | Sample 2   | 0.337                    | 1.06  | 2.53  | 4.66  |
|                       | Mean       | 0.363                    | 1.07  | 2.54  | 4.91  |
|                       | Percent RE | -9.37                    | +6.50 | +1.40 | -1.90 |

**TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA**

PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -20°C

| Paromomycin           |            | CONCENTRATION<br>( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|---------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                                 | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                                       |       |       |       |
| 2 months              | Sample 1   | 0.327                                 | 0.701 | 1.96  | 3.34  |
|                       | Sample 2   | 0.325                                 | 0.811 | 1.77  | 3.42  |
|                       | Mean       | 0.326                                 | 0.756 | 1.87  | 3.38  |
|                       | Percent RE | -18.5                                 | -24.4 | -25.4 | -32.4 |
| 3 months              | Sample 1   | 0.265                                 | 0.675 | 1.57  | 2.93  |
|                       | Sample 2   | 0.292                                 | 0.659 | 1.57  | 2.98  |
|                       | Mean       | 0.279                                 | 0.667 | 1.57  | 2.96  |
|                       | Percent RE | -30.4                                 | -33.3 | -37.2 | -40.9 |
| 6 months              | Sample 1   | 0.326                                 | 0.627 | 1.78  | 2.85  |
|                       | Sample 2   | 0.334                                 | 0.668 | 1.66  | 3.20  |
|                       | Mean       | 0.330                                 | 0.648 | 1.72  | 3.03  |
|                       | Percent RE | -17.5                                 | -35.3 | -31.2 | -39.5 |
| 1 year                | Sample 1   | 0.228                                 | 0.471 | 1.10  | 1.67  |
|                       | Sample 2   | 0.181                                 | 0.496 | 1.31  | 2.15  |
|                       | Mean       | 0.205                                 | 0.484 | 1.21  | 1.91  |
|                       | Percent RE | -48.9                                 | -51.7 | -51.8 | -61.8 |

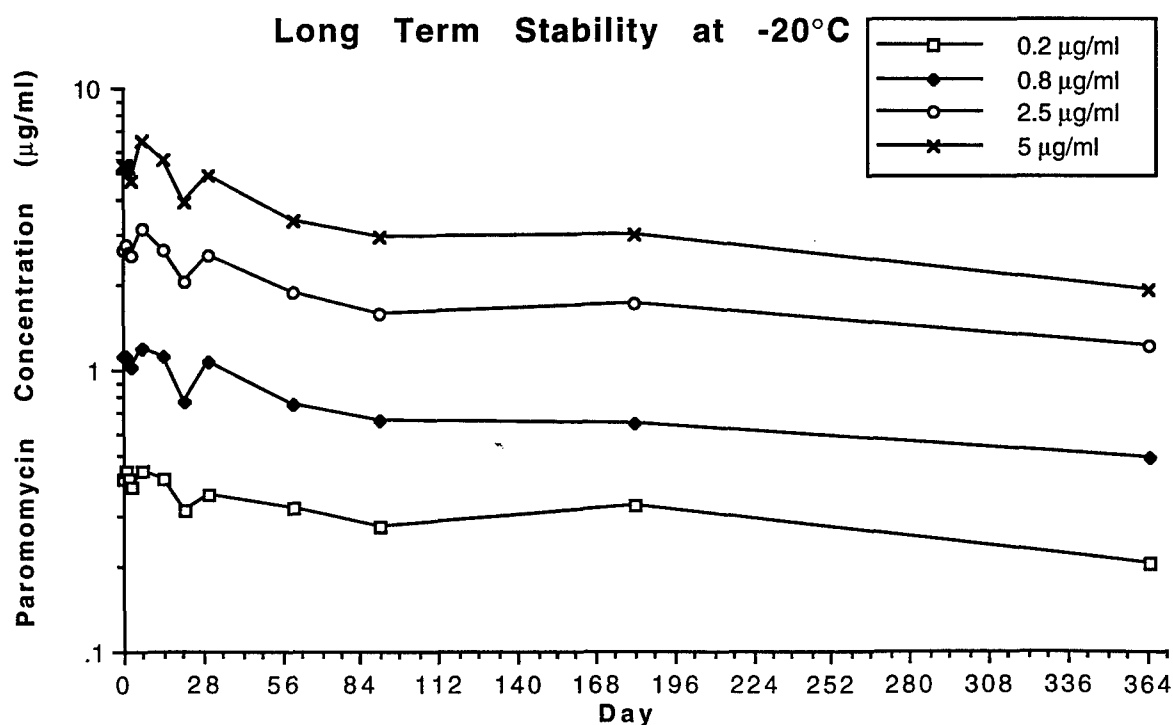


**TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA**

**PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -70°C**

| Paromomycin           |            | CONCENTRATION<br>( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|---------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                                 | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                                       |       |       |       |
| 0 days                | Sample 1   | 0.415                                 | 1.07  | 2.45  | 5.03  |
|                       | Sample 2   | 0.404                                 | 1.07  | 2.63  | 5.18  |
|                       | Mean       | 0.410                                 | 1.07  | 2.54  | 5.11  |
|                       | Percent RE | +2.37                                 | +7.00 | +1.60 | +2.10 |
| 1 day                 | Sample 1   | 0.474                                 | 1.26  | 3.06  | 6.46  |
|                       | Sample 2   | 0.482                                 | 1.22  | 2.96  | 5.96  |
|                       | Mean       | 0.478                                 | 1.24  | 3.01  | 6.21  |
|                       | Percent RE | +19.5                                 | +24.0 | +20.4 | +24.2 |
| 2 days                | Sample 1   | 0.589                                 | 1.45  | 3.47  | 6.98  |
|                       | Sample 2   | 0.528                                 | 1.34  | 3.36  | 6.74  |
|                       | Mean       | 0.559                                 | 1.40  | 3.42  | 6.86  |
|                       | Percent RE | +39.6                                 | +39.5 | +36.6 | +37.2 |
| 3 days                | Sample 1   | 0.414                                 | 1.11  | 2.83  | 5.76  |
|                       | Sample 2   | 0.420                                 | 1.08  | 2.72  | 5.52  |
|                       | Mean       | 0.417                                 | 1.10  | 2.78  | 5.64  |
|                       | Percent RE | +4.25                                 | +9.50 | +11.0 | +12.8 |
| 1 week                | Sample 1   | 0.484                                 | 1.39  | 3.62  | 7.19  |
|                       | Sample 2   | 0.447                                 | 1.34  | 3.49  | 6.58  |
|                       | Mean       | 0.466                                 | 1.37  | 3.56  | 6.89  |
|                       | Percent RE | +16.4                                 | +36.5 | +42.2 | +37.7 |
| 2 weeks               | Sample 1   | 0.413                                 | 1.09  | 2.68  | 5.59  |
|                       | Sample 2   | 0.423                                 | 1.06  | 2.65  | 5.45  |
|                       | Mean       | 0.418                                 | 1.08  | 2.67  | 5.52  |
|                       | Percent RE | +4.50                                 | +7.50 | +6.60 | +10.4 |
| 3 weeks               | Sample 1   | 0.377                                 | 0.972 | 2.40  | 4.44  |
|                       | Sample 2   | 0.377                                 | 1.01  | 2.37  | 4.65  |
|                       | Mean       | 0.377                                 | 0.991 | 2.39  | 4.55  |
|                       | Percent RE | -5.75                                 | -0.90 | -4.60 | -9.10 |
| 1 month               | Sample 1   | 0.361                                 | 0.953 | 2.43  | 5.13  |
|                       | Sample 2   | 0.370                                 | 0.983 | 2.40  | 4.94  |
|                       | Mean       | 0.366                                 | 0.968 | 2.42  | 5.04  |
|                       | Percent RE | -8.63                                 | -3.20 | -3.40 | +0.70 |

**TABLE 7B: LONG TERM STABILITY OF PAROMOMYCIN IN HUMAN PLASMA**

**PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT -70°C**

| Paromomycin           |            | CONCENTRATION<br>( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|---------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.400                                 | 1.00  | 2.50  | 5.00  |
| TIME STORED           |            |                                       |       |       |       |
| 2 months              | Sample 1   | 0.334                                 | 0.932 | 3.04  | 5.39  |
|                       | Sample 2   | 0.342                                 | 0.933 | 2.53  | 5.05  |
|                       | Mean       | 0.338                                 | 0.933 | 2.79  | 5.22  |
|                       | Percent RE | -15.5                                 | -6.75 | +11.4 | +4.40 |
| 3 months              | Sample 1   | 0.390                                 | 0.991 | 2.52  | 5.07  |
|                       | Sample 2   | 0.362                                 | 1.01  | 2.49  | 4.96  |
|                       | Mean       | 0.376                                 | 1.00  | 2.51  | 5.02  |
|                       | Percent RE | -6.00                                 | +0.05 | +0.20 | +0.30 |
| 6 months              | Sample 1   | 0.315                                 | 0.951 | 2.35  | 4.87  |
|                       | Sample 2   | 0.309                                 | 0.918 | 2.36  | 4.75  |
|                       | Mean       | 0.312                                 | 0.935 | 2.36  | 4.81  |
|                       | Percent RE | -22.0                                 | -6.55 | -5.80 | -3.80 |
| 1 year                | Sample 1   | 0.376                                 | 1.05  | 2.70  | 5.59  |
|                       | Sample 2   | 0.378                                 | 1.02  | 2.72  | 5.60  |
|                       | Mean       | 0.377                                 | 1.04  | 2.71  | 5.60  |
|                       | Percent RE | -5.8                                  | +3.50 | +8.4  | +11.9 |

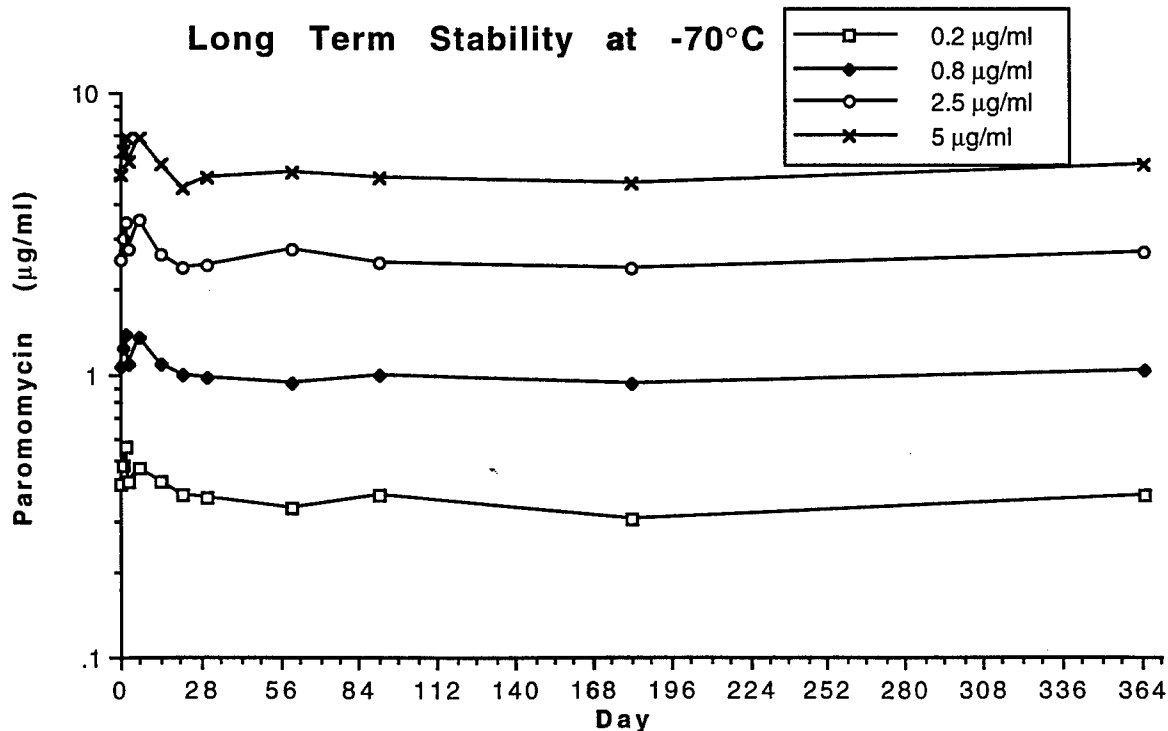


**TABLE 8A: BENCH TOP STABILITY OF GENTAMICIN IN SPIKED HUMAN PLASMA**

**GENTAMICIN CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE**

| Spiked Concentration: |            | CONCENTRATION ( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|------------------------------------|-------|-------|-------|
|                       |            | 0.200                              | 0.800 | 2.50  | 5.00  |
| TIME STORED           |            |                                    |       |       |       |
| 0 hours               | Sample 1   | 0.219                              | 0.798 | 2.56  | 5.01  |
|                       | Sample 2   | 0.190                              | 0.843 | 2.69  | 4.84  |
|                       | Mean       | 0.205                              | 0.821 | 2.63  | 4.93  |
|                       | Percent RE | +2.50                              | +2.62 | +5.20 | -1.40 |
| 2 hours               | Sample 1   | 0.196                              | 0.758 | 2.43  | 5.01  |
|                       | Sample 2   | 0.219                              | 0.805 | 2.71  | 5.39  |
|                       | Mean       | 0.208                              | 0.782 | 2.57  | 5.20  |
|                       | Percent RE | 4.00                               | -2.25 | +2.80 | +4.00 |
| 4 hours               | Sample 1   | 0.190                              | 0.776 | 2.72  | 5.41  |
|                       | Sample 2   | 0.266                              | 0.789 | 2.67  | 5.10  |
|                       | Mean       | 0.228                              | 0.783 | 2.70  | 5.26  |
|                       | Percent RE | 14.0                               | -2.13 | +8.00 | +5.20 |
| 6 hours               | Sample 1   | 0.168                              | 0.751 | 2.55  | 5.14  |
|                       | Sample 2   | 0.228                              | 0.954 | 2.71  | 5.29  |
|                       | Mean       | 0.198                              | 0.853 | 2.63  | 5.22  |
|                       | Percent RE | -1.00                              | +6.62 | +5.20 | +4.40 |

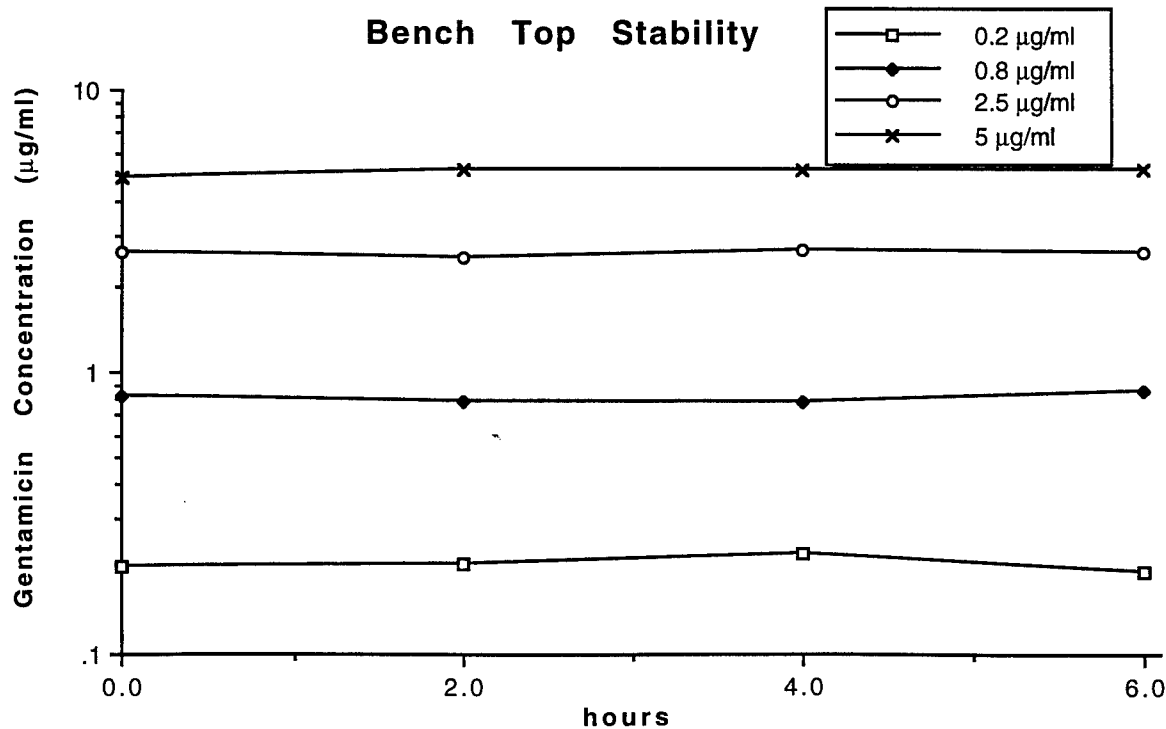


**TABLE 8B: BENCH TOP STABILITY OF PAROMOMYCIN IN SPIKED HUMAN PLASMA**

PAROMOMYCIN CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE

|                       |            | CONCENTRATION ( $\mu\text{g/ml}$ ) |       |       |       |
|-----------------------|------------|------------------------------------|-------|-------|-------|
| Spiked Concentration: |            | 0.200                              | 0.800 | 2.50  | 5.00  |
| TIME STORED           |            |                                    |       |       |       |
| 0 hours               | Sample 1   | 0.232                              | 0.910 | 2.91  | 5.67  |
|                       | Sample 2   | 0.171                              | 0.858 | 2.66  | 4.85  |
|                       | Mean       | 0.202                              | 0.884 | 2.79  | 5.26  |
|                       | Percent RE | +1.00                              | +10.5 | +11.6 | +5.20 |
| 2 hours               | Sample 1   | 0.182                              | 0.791 | 2.74  | 5.62  |
|                       | Sample 2   | 0.229                              | 0.929 | 2.66  | 5.36  |
|                       | Mean       | 0.206                              | 0.860 | 2.70  | 5.49  |
|                       | Percent RE | +3.00                              | +7.50 | +8.00 | +9.80 |
| 4 hours               | Sample 1   | 0.179                              | 0.814 | 2.73  | 5.48  |
|                       | Sample 2   | 0.285                              | 0.929 | 3.12  | 5.92  |
|                       | Mean       | 0.232                              | 0.872 | 2.93  | 5.70  |
|                       | Percent RE | +16.0                              | +9.00 | +17.2 | +14.0 |
| 6 hours               | Sample 1   | 0.171                              | 0.764 | 2.50  | 5.05  |
|                       | Sample 2   | 0.245                              | 1.10  | 3.18  | 6.15  |
|                       | Mean       | 0.208                              | 0.932 | 2.84  | 5.60  |
|                       | Percent RE | +4.00                              | +16.5 | +13.6 | +12.0 |

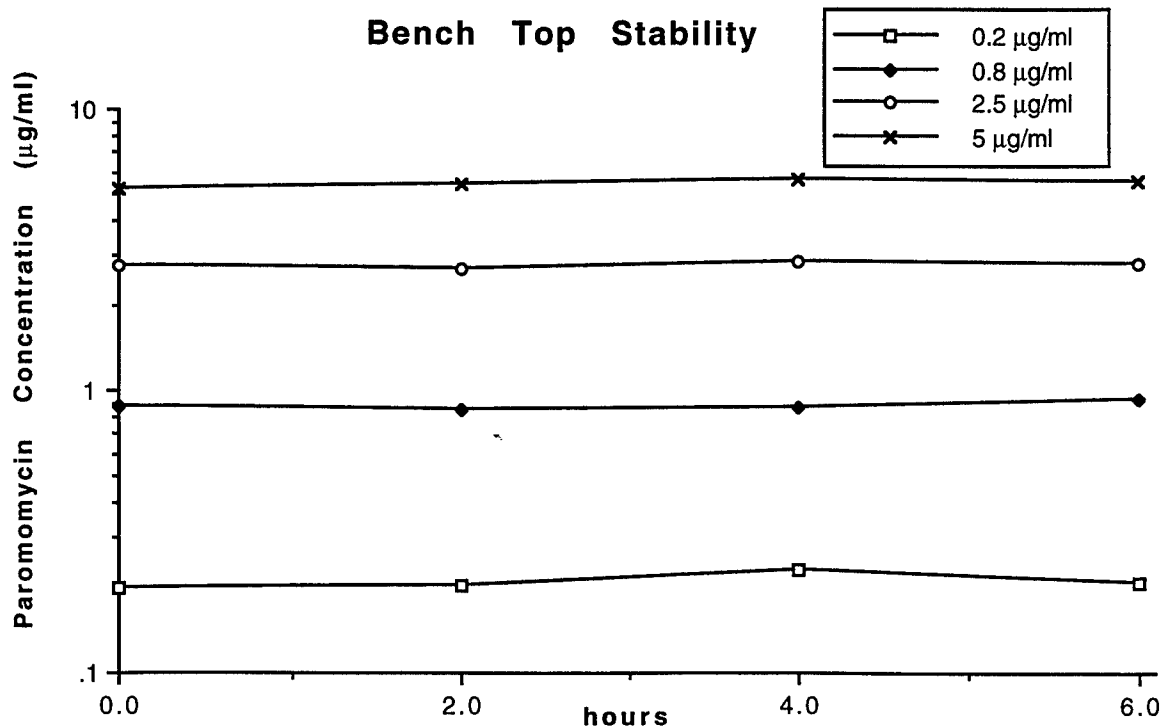


**TABLE 9A: EFFECT OF REPEATED FREEZE (-70°C) AND THAW CYCLES ON GENTAMICIN SPIKED HUMAN PLASMA**

| Gentamicin            |            | CONCENTRATION ( $\mu\text{g/ml}$ ) |        |
|-----------------------|------------|------------------------------------|--------|
| Spiked Concentration: |            | 0.800                              | 5.00   |
| CYCLE                 |            |                                    |        |
| 1                     | Sample 1   | 0.891                              | 5.12   |
|                       | Sample 2   | 0.823                              | 4.98   |
|                       | Mean       | 0.857                              | 5.05   |
|                       | Percent RE | 7.12                               | +1.00  |
| 2                     | Sample 1   | 0.808                              | 5.28   |
|                       | Sample 2   | 0.763                              | 4.67   |
|                       | Mean       | 0.786                              | 4.98   |
|                       | Percent RE | -1.75                              | -0.400 |
| 3                     | Sample 1   | 0.750                              | 5.29   |
|                       | Sample 2   | 0.695                              | 5.12   |
|                       | Mean       | 0.723                              | 5.21   |
|                       | Percent RE | -9.63                              | +4.20  |
| 4                     | Sample 1   | 0.727                              | 5.18   |
|                       | Sample 2   | 0.669                              | 5.90   |
|                       | Mean       | 0.698                              | 5.54   |
|                       | Percent RE | -12.8                              | +10.8  |
| 5                     | Sample 1   | 0.616                              | 5.20   |
|                       | Sample 2   | 0.684                              | 4.79   |
|                       | Mean       | 0.650                              | 5.00   |
|                       | Percent RE | -18.8                              | 0      |

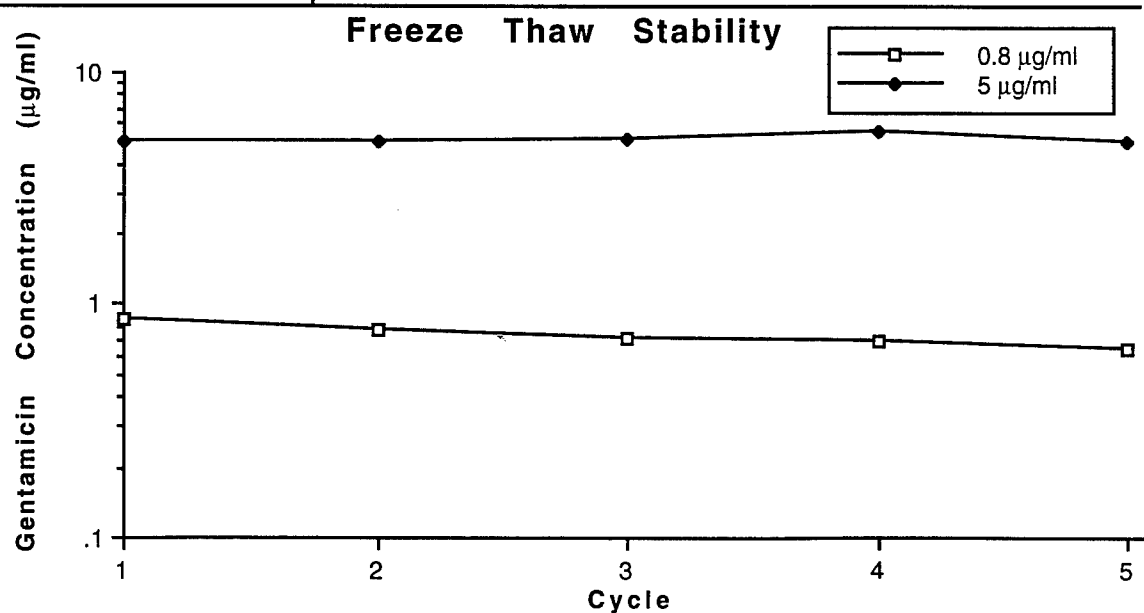


**TABLE 9B: EFFECT OF REPEATED FREEZE (-70°C) AND THAW CYCLES ON PAROMOMYCIN SPIKED HUMAN PLASMA**

| Paromomycin           |            | CONCENTRATION ( $\mu\text{g/ml}$ ) |        |
|-----------------------|------------|------------------------------------|--------|
| Spiked Concentration: |            | 0.800                              | 5.00   |
| CYCLE                 |            |                                    |        |
| 1                     | Sample 1   | 0.855                              | 4.94   |
|                       | Sample 2   | 0.758                              | 4.99   |
|                       | Mean       | 0.807                              | 4.97   |
|                       | Percent RE | +0.875                             | -0.600 |
| 2                     | Sample 1   | 0.714                              | 5.12   |
|                       | Sample 2   | 0.701                              | 4.57   |
|                       | Mean       | 0.708                              | 4.85   |
|                       | Percent RE | -11.5                              | -3.00  |
| 3                     | Sample 1   | 0.658                              | 5.51   |
|                       | Sample 2   | 0.639                              | 4.96   |
|                       | Mean       | 0.649                              | 5.24   |
|                       | Percent RE | -18.9                              | +4.80  |
| 4                     | Sample 1   | 0.684                              | 4.98   |
|                       | Sample 2   | 0.651                              | 5.76   |
|                       | Mean       | 0.668                              | 5.37   |
|                       | Percent RE | -16.5                              | +7.40  |
| 5                     | Sample 1   | 0.639                              | 5.32   |
|                       | Sample 2   | 0.681                              | 4.87   |
|                       | Mean       | 0.660                              | 5.10   |
|                       | Percent RE | -17.5                              | +2.00  |

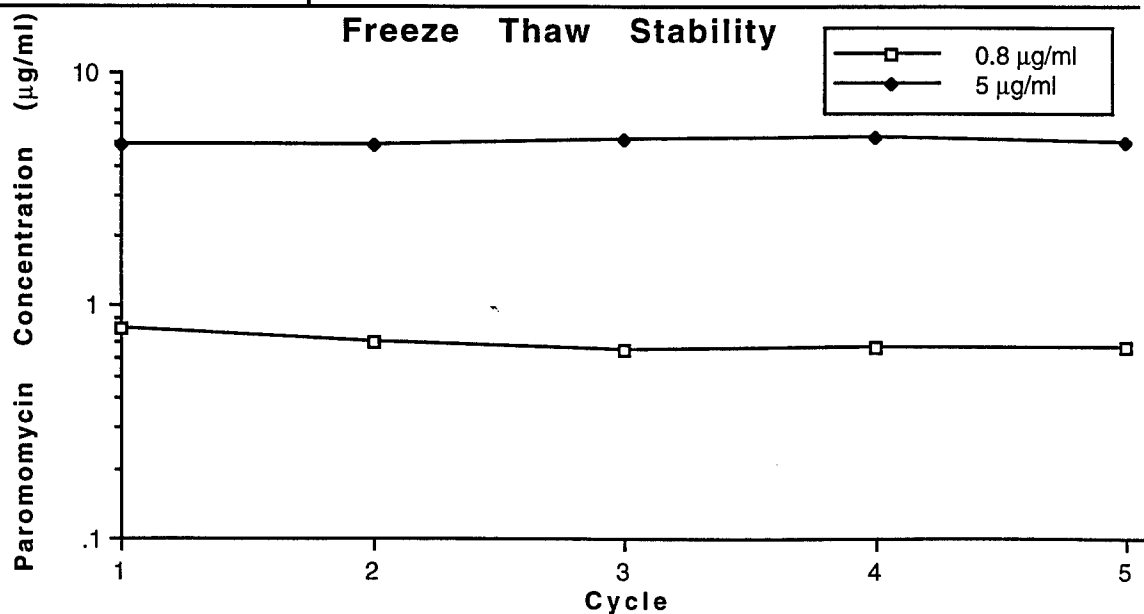


**TABLE 10A: ACCURACY OF GENTAMICIN HUMAN PLASMA ASSAY  
(BLIND STUDY RESULTS)**

Gentamicin free base

| Sample Number | Spiked Level (µg/ml) | Measured Level# (µg/ml) | Statistics (µg/ml) |         |
|---------------|----------------------|-------------------------|--------------------|---------|
| 2             | 0                    | 0                       |                    |         |
| 9             |                      | 0                       |                    |         |
| 16            |                      | 0                       |                    |         |
| 17            |                      | 0                       |                    |         |
| 20            |                      | 0                       |                    |         |
| 24            |                      | 0                       |                    |         |
| 8             | 0.2                  | 0.211                   | Mean =             | 0.199   |
| 12            |                      | 0.203                   | SD =               | 0.00844 |
| 13            |                      | 0.200                   | Percent CV =       | 4.23    |
| 15            |                      | 0.194                   | Percent Bias =     | -0.3    |
| 18            |                      | 0.189                   |                    |         |
| 3             | 0.4                  | 0.385                   | Mean =             | 0.402   |
| 7             |                      | 0.378                   | SD =               | 0.0336  |
| 11            |                      | 0.394                   | Percent CV =       | 8.35    |
| 22            |                      | 0.461                   | Percent Bias =     | 0.5     |
| 23            |                      | 0.392                   |                    |         |
| 1             | 1                    | 0.831                   | Mean =             | 0.806   |
| 4             |                      | 0.774                   | SD =               | 0.027   |
| 10            |                      | 0.834                   | Percent CV =       | 3.35    |
| 21            |                      | 0.784                   | Percent Bias =     | -19.4   |
| 26            |                      | 0.806                   |                    |         |
| 5             | 8                    | 5.86                    | Mean =             | 5.93    |
| 6             |                      | 5.72                    | SD =               | 0.28    |
| 14            |                      | 5.63                    | Percent CV =       | 4.73    |
| 19            |                      | 6.25                    | Percent Bias =     | -25.9   |
| 25            |                      | 6.20                    |                    |         |

# Measured concentrations are averages of three analyses.

**TABLE 10B: ACCURACY OF PAROMOMYCIN HUMAN PLASMA ASSAY  
(BLIND STUDY RESULTS)**

Paromomycin free base

| Sample Number | Spiked Level (µg/ml) | Measured Level <sup>#</sup> (µg/ml) | Statistics (µg/ml) |        |
|---------------|----------------------|-------------------------------------|--------------------|--------|
| 5             | 0                    | 0                                   |                    |        |
| 9             |                      | 0                                   |                    |        |
| 10            |                      | 0                                   |                    |        |
| 13            |                      | 0                                   |                    |        |
| 22            |                      | 0                                   |                    |        |
| 26            |                      | 0                                   |                    |        |
| 3             | 0.2                  | 0.215                               | Mean =             | 0.205  |
| 14            |                      | 0.177                               | SD =               | 0.0167 |
| 18            |                      | 0.208                               | Percent CV =       | 8.16   |
| 20            |                      | 0.205                               | Percent Bias =     | 2.5    |
| 21            |                      | 0.220                               |                    |        |
| 6             | 0.5                  | 0.557                               | Mean =             | 0.498  |
| 11            |                      | 0.495                               | SD =               | 0.0337 |
| 15            |                      | 0.482                               | Percent CV =       | 6.76   |
| 17            |                      | 0.475                               | Percent Bias =     | -0.36  |
| 24            |                      | 0.482                               |                    |        |
| 4             | 3                    | 3.03                                | Mean =             | 2.96   |
| 7             |                      | 2.97                                | SD =               | 0.101  |
| 12            |                      | 2.94                                | Percent CV =       | 3.42   |
| 16            |                      | 2.88                                | Percent Bias =     | -1.33  |
| 25            |                      | 3.06                                |                    |        |
| 1             | 9                    | 9.15                                | Mean =             | 8.71   |
| 2             |                      | 8.65                                | SD =               | 0.258  |
| 8             |                      | 8.49                                | Percent CV =       | 2.97   |
| 19            |                      | 8.57                                | Percent Bias =     | -3.27  |
| 23            |                      | 8.67                                |                    |        |

<sup>#</sup> Measured concentrations are averages of three analyses.

**TABLE 11: PRECISION STANDARD CURVE DATA FOR GENTAMICIN/  
PAROMOMYCIN RAT PLASMA ASSAY, STUDY REPORT 24**

Gentamicin Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|----------------|------------|------------|---------------------------------|
| 3/1/95                 | rintrage       | 0.44400042 | -0.0050161 | 0.99988014                      |
| 3/8/95                 | ginte3ra       | 0.45454769 | 0.00537975 | 0.99948715                      |
| 3/9/95                 | rtinte3g       | 0.42375694 | 0.01053217 | 0.99970313                      |

Gentamicin Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |         |        |        |        |        |        |        |
|-------------------|---------------------------------------|---------|--------|--------|--------|--------|--------|--------|
|                   | 0.100                                 | 0.200   | 0.400  | 0.800  | 1.50   | 3.00   | 6.00   | 12.0   |
|                   | Back Calculated Concentration (µg/ml) |         |        |        |        |        |        |        |
| rintrage          | 0.0946                                | 0.205   | 0.426  | 0.788  | 1.5    | 2.98   | 6.02   | 12     |
| ginte3ra          | 0.1                                   | 0.197   | 0.391  | 0.842  | 1.46   | 3.1    | 5.86   | 12.1   |
| rtinte3g          | 0.103                                 | 0.195   | 0.402  | 0.827  | 1.47   | 3      | 5.87   | 12.1   |
| n 3               | 3                                     | 3       | 3      | 3      | 3      | 3      | 3      |        |
| Mean              | 0.0992                                | 0.199   | 0.406  | 0.819  | 1.48   | 3.03   | 5.92   | 12.1   |
| SD                | 0.00426                               | 0.00529 | 0.0179 | 0.0279 | 0.0208 | 0.0643 | 0.0896 | 0.0577 |
| Percent CV        | 4.29                                  | 2.66    | 4.4    | 3.4    | 1.41   | 2.12   | 1.51   | 0.478  |
| Percent RE        | -0.8                                  | -0.5    | 1.58   | 2.37   | -1.56  | 0.889  | -1.39  | 0.556  |

Paromomycin Standard Curve Parameters

| Validation Run<br>Date | Validation Run | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|----------------|------------|------------|---------------------------------|
| 3/1/95                 | rintrapa       | 1.54571936 | 0.05064803 | 0.99960491                      |
| 3/8/95                 | pinte3rt       | 1.61517291 | 0.01253853 | 0.9988574                       |
| 3/9/95                 | rainte3p       | 1.3005778  | 0.02468605 | 0.99967686                      |

Paromomycin Back Calculated Standard Calibrators

| Validation<br>Run | Spiked Concentration (µg/ml)          |        |        |        |         |      |       |        |
|-------------------|---------------------------------------|--------|--------|--------|---------|------|-------|--------|
|                   | 0.100                                 | 0.200  | 0.400  | 0.800  | 1.50    | 3.00 | 6.00  | 12.0   |
|                   | Back Calculated Concentration (µg/ml) |        |        |        |         |      |       |        |
| rintrapa          | 0.0863                                | 0.204  | 0.425  | 0.806  | 1.53    | 3.08 | 6.05  | 11.8   |
| pinte3rt          | 0.087                                 | 0.208  | 0.403  | 0.886  | 1.52    | 3.06 | 5.75  | 12.1   |
| rainte3p          | 0.104                                 | 0.186  | 0.389  | 0.86   | 1.52    | 2.95 | 6     | 12     |
| n 3               | 3                                     | 3      | 3      | 3      | 3       | 3    | 3     |        |
| Mean              | 0.0924                                | 0.199  | 0.406  | 0.851  | 1.52    | 3.03 | 5.93  | 12     |
| SD                | 0.01                                  | 0.0117 | 0.0181 | 0.0408 | 0.00577 | 0.07 | 0.161 | 0.153  |
| Percent CV        | 10.8                                  | 5.88   | 4.47   | 4.8    | 0.379   | 2.31 | 2.71  | 1.28   |
| Percent RE        | -7.57                                 | -0.333 | 1.42   | 6.33   | 1.56    | 1    | -1.11 | -0.278 |

TABLE 12A: PRECISION OF GENTAMICIN RAT PLASMA ASSAY

Interday Precision Gentamicin

| Validation<br>Run               | QC<br>Sample No. | Spiked Concentrations (µg/mL) |        |       |       |
|---------------------------------|------------------|-------------------------------|--------|-------|-------|
|                                 |                  | 0.200                         | 0.800  | 2.50  | 5.00  |
| Measured Concentrations (µg/mL) |                  |                               |        |       |       |
| rintrage                        | 1                | 0.2                           | 0.829  | 2.08  | 4.47  |
|                                 | 2                | 0.191                         | 0.811  | 2.29  | 4.64  |
| ginte3ra                        | 1                | 0.221                         | 0.855  | 2.55  | 4.57  |
|                                 | 2                | 0.199                         | 0.87   | 2.61  | 4.75  |
| rtinte3g                        | 1                | 0.188                         | 0.87   | 2.47  | 4.72  |
|                                 | 2                | 0.221                         | 0.803  | 2.63  | 5.07  |
| n                               |                  | 6                             | 6      | 6     | 6     |
| Mean                            |                  | 0.203                         | 0.84   | 2.44  | 4.7   |
| SD                              |                  | 0.0144                        | 0.0295 | 0.215 | 0.206 |
| Percent CV                      |                  | 7.1                           | 3.51   | 8.8   | 4.39  |
| Percent RE                      |                  | 1.67                          | 4.96   | -2.47 | -5.93 |

Intraday Precision Gentamicin

| Validation                      | QC         | Spiked Concentrations (µg/mL) |        |       |       |
|---------------------------------|------------|-------------------------------|--------|-------|-------|
| Run                             | Sample No. | 0.200                         | 0.800  | 2.50  | 5.00  |
| Measured Concentrations (µg/mL) |            |                               |        |       |       |
| rintrage                        | 1          | 0.216                         | 0.802  | 2.2   | 4.71  |
|                                 | 2          | 0.209                         | 0.784  | 2.47  | 4.7   |
|                                 | 3          | 0.194                         | 0.829  | 2.26  | 4.61  |
|                                 | 4          | 0.203                         | 0.804  | 2.28  | 4.62  |
|                                 | 5          | 0.212                         | 0.802  | 2.21  | 4.86  |
|                                 | 6          | 0.2                           | 0.838  | 2.19  | 4.68  |
| n                               | 6          | 6                             | 6      | 6     |       |
| Mean                            |            | 0.206                         | 0.81   | 2.27  | 4.7   |
| SD                              |            | 0.00816                       | 0.0199 | 0.105 | 0.09  |
| Percent CV                      |            | 3.97                          | 2.46   | 4.63  | 1.92  |
| Percent RE                      |            | 2.83                          | 1.23   | -9.27 | -6.07 |

TABLE 12B: PRECISION OF PAROMOMYCIN RAT PLASMA ASSAY

Interday Precision Paromomycin

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (µg/mL) |        |       |       |
|---------------------------------|------------------|-------------------------------|--------|-------|-------|
|                                 |                  | 0.200                         | 0.800  | 2.50  | 5.00  |
| Measured Concentrations (µg/mL) |                  |                               |        |       |       |
| rintrapa                        | 1                | 0.201                         | 0.899  | 2.01  | 4.7   |
|                                 | 2                | 0.187                         | 0.882  | 2.27  | 4.74  |
| pinte3rt                        | 1                | 0.189                         | 0.806  | 2.41  | 4.2   |
|                                 | 2                | 0.224                         | 0.989  | 2.89  | 4.93  |
| rainte3p                        | 1                | 0.177                         | 0.902  | 2.57  | 4.93  |
|                                 | 2                | 0.206                         | 0.833  | 2.63  | 5.03  |
| n                               |                  | 6                             | 6      | 6     | 6     |
| Mean                            |                  | 0.197                         | 0.885  | 2.46  | 4.76  |
| SD                              |                  | 0.0167                        | 0.0636 | 0.306 | 0.299 |
| Percent CV                      |                  | 8.45                          | 7.19   | 12.4  | 6.3   |
| Percent RE                      |                  | -1.33                         | 10.6   | -1.47 | -4.9  |

Intraday Precision Paromomycin

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (µg/mL) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 0.200                         | 0.800 | 2.50  | 5.00  |
| Measured Concentrations (µg/mL) |                  |                               |       |       |       |
| rintrapa                        | 1                | 0.21                          | 0.905 | 2.38  | 5.07  |
|                                 | 2                | 0.214                         | 0.934 | 2.84  | 5.25  |
|                                 | 3                | 0.203                         | 0.971 | 2.23  | 5.32  |
|                                 | 4                | 0.166                         | 0.791 | 2.19  | 4.5   |
|                                 | 5                | 0.17                          | 0.766 | 2.07  | 4.69  |
|                                 | 6                | 0.144                         | 0.746 | 1.93  | 4.3   |
|                                 | n                | 6                             | 6     | 6     | 6     |
|                                 | Mean             | 0.185                         | 0.852 | 2.27  | 4.86  |
|                                 | SD               | 0.0285                        | 0.096 | 0.316 | 0.419 |
|                                 | Percent CV       | 15.4                          | 11.3  | 13.9  | 8.64  |
|                                 | Percent RE       | -7.75                         | 6.52  | -9.07 | -2.9  |

TABLE 13: LOWER LIMIT OF QUANTITATION OF THE RAT PLASMA  
ASSAY FOR GENTAMICIN/PAROMOMYCIN

| Gentamicin           |                                    |             |  |
|----------------------|------------------------------------|-------------|--|
| Spiked Concentration | 0.100 µg/ml                        | 0.100 µg/ml |  |
| Sample               | Measured Concentrations<br>(µg/ml) |             |  |
|                      | Interday                           | Intraday    |  |
| 1                    | 0.0946                             | 0.0929      |  |
| 2                    | 0.1                                | 0.0974      |  |
| 3                    | 0.103                              | 0.0861      |  |
| 4                    | -                                  | 0.0996      |  |
| 5                    | -                                  | 0.0906      |  |
| 6                    | -                                  | 0.0974      |  |
| Mean                 | 0.0992                             | 0.094       |  |
| SD                   | 0.00426                            | 0.00509     |  |
| Percent CV           | 4.29                               | 5.42        |  |
| Percent RE           | -0.8                               | -6          |  |

| Paromomycin          |                                    |             |  |
|----------------------|------------------------------------|-------------|--|
| Spiked Concentration | 0.100 µg/ml                        | 0.100 µg/ml |  |
| Sample               | Measured Concentrations<br>(µg/ml) |             |  |
|                      | Interday                           | Intraday    |  |
| 1                    | 0.0863                             | 0.0834      |  |
| 2                    | 0.087                              | 0.0877      |  |
| 3                    | 0.104                              | 0.0758      |  |
| 4                    | -                                  | 0.0958      |  |
| 5                    | -                                  | 0.0839      |  |
| 6                    | -                                  | 0.0915      |  |
| Mean                 | 0.0924                             | 0.0864      |  |
| SD                   | 0.01                               | 0.00698     |  |
| Percent CV           | 10.8                               | 8.08        |  |
| Percent RE           | -7.57                              | -13.7       |  |

TABLE 14: RECOVERY OF GENTAMICIN/PAROMOMYCIN FROM RAT PLASMA

| SAMPLE ID                        | SPIKED CONCENTRATION Range | SPIKED CONCENTRATION (µg/ml) | PEAK HEIGHT RATIO |              | MEAN PERCENT RECOVERY |
|----------------------------------|----------------------------|------------------------------|-------------------|--------------|-----------------------|
|                                  |                            |                              | SOLVENT           | PLASMA       |                       |
| Gentamicin                       |                            |                              |                   |              |                       |
| 1                                | X Low                      | 0.200                        | 0.051             | 0.048        | 95.3                  |
| 2                                |                            |                              | 0.049             | 0.045        |                       |
| 3                                |                            |                              | 0.049             | 0.049        |                       |
| Mean (± SD)                      |                            |                              | 0.050 ±0.001      | 0.047 ±0.002 |                       |
| 1                                | Low                        | 0.800                        | 0.186             | 0.177        | 96.0                  |
| 2                                |                            |                              | 0.178             | 0.176        |                       |
| 3                                |                            |                              | 0.187             | 0.176        |                       |
| Mean (± SD)                      |                            |                              | 0.184 ±0.005      | 0.176 ±0.001 |                       |
| 1                                | Medium                     | 2.50                         | 0.576             | 0.523        | 89.3                  |
| 2                                |                            |                              | 0.579             | 0.514        |                       |
| 3                                |                            |                              | 0.58              | 0.512        |                       |
| Mean (± SD)                      |                            |                              | 0.578 ±0.002      | 0.516 ±0.006 |                       |
| 1                                | High                       | 5.00                         | 1.137             | 0.998        | 93.2                  |
| 2                                |                            |                              | 1.136             | 1.051        |                       |
| 3                                |                            |                              | 1.121             | 1.114        |                       |
| Mean (± SD)                      |                            |                              | 1.131 ±0.009      | 1.054 ±0.058 |                       |
| AVERAGE =                        |                            |                              |                   |              | 93.4                  |
| Paromomycin                      |                            |                              |                   |              |                       |
| 1                                | X Low                      | 0.200                        | 0.21              | 0.128        | 69.7                  |
| 2                                |                            |                              | 0.198             | 0.136        |                       |
| 3                                |                            |                              | 0.196             | 0.157        |                       |
| Mean (± SD)                      |                            |                              | 0.201 ±0.008      | 0.140 ±0.015 |                       |
| 1                                | Low                        | 0.800                        | 0.805             | 0.545        | 68.5                  |
| 2                                |                            |                              | 0.829             | 0.544        |                       |
| 3                                |                            |                              | 0.789             | 0.571        |                       |
| Mean (± SD)                      |                            |                              | 0.808 ±0.020      | 0.553 ±0.015 |                       |
| 1                                | Medium                     | 2.50                         | 2.44              | 1.592        | 65.2                  |
| 2                                |                            |                              | 2.467             | 1.546        |                       |
| 3                                |                            |                              | 2.422             | 1.644        |                       |
| Mean (± SD)                      |                            |                              | 2.443 ±0.023      | 1.594 ±0.049 |                       |
| 1                                | High                       | 5.00                         | 4.709             | 3.117        | 69.5                  |
| 2                                |                            |                              | 4.867             | 3.167        |                       |
| 3                                |                            |                              | 4.654             | 3.603        |                       |
| Mean (± SD)                      |                            |                              | 4.743 ±0.111      | 3.296 ±0.267 |                       |
| AVERAGE =                        |                            |                              |                   |              | 68.2                  |
| b.c. = unacceptable chromatogram |                            |                              |                   |              |                       |

**LABORATORY METHODOLOGY FOR PYRIDOSTIGMINE (CATION)  
PLASMA ASSAY,\* STUDY REPORT 25**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 710B (Waters Associates, Milford, MA) or equivalent.
2. LC-600 Shimadzu Pump (Shimadzu Corp., Kyoto, Japan) or equivalent.
3. Shimadzu SPD 10A UV Detector (Shimadzu Corp., Kyoto, Japan) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. All solvents are HPLC grade unless otherwise specified.
2. All chemicals are reagent grade unless otherwise specified.
3. Pyridostigmine bromide, lot no. 8950426 (Sigma). The certificate of analysis lists the chemical formula as  $C_9H_{13}BrN_2O_2$  and the purity as 99% by thin layer chromatography.
4. Neostigmine bromide, lot no. KT05130J (Aldrich Chemicals).
5. Tetramethylammonium chloride (TMACl) (Fluka Chemika).
6. Acetonitrile (Fisher Scientific, Fair Lawn, NJ).
7. Phosphoric acid (Fisher Scientific, Fair Lawn, NJ).
8. Water (deionized by Nanopure II, Barnstead Co., Boston, MA).
9. Dibasic ammonium phosphate (Fisher Scientific, Fair Lawn, NJ).

\*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

### C. ASSAY CONDITIONS

#### 1. DETECTOR

##### Settings

Wavelength; 208 nm  
Sensitivity: 0.003 aufs  
Rise Time: 1.0 s

##### Lamp

Deuterium Lamp -2900-0484, ABI Analytical, Inc., Ramsey, NJ.

#### 2. COLUMN

Axxiom Silica, 5  $\mu$ m particle size, 4.6 x 250 mm (Richard Scientific, Novato, CA) or equivalent.

#### 3. SOLVENT SYSTEM

CH<sub>3</sub>CN/H<sub>2</sub>O (1:1, v/v) with 0.05% TMACl, 5 mM (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>  
(final concentrations) apparent pH = 7.2

#### 4. FLOW RATE

1.0 ml/min

#### 5. REPRESENTATIVE STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and checked for deterioration by comparison to a newly made solution (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 6 months after the preparation date).

##### a. Pyridostigmine bromide for interday plasma precision expressed as the cation concentration.

Prep date: 11/23/94

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent**  | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|------------|---------------------|
| Standard Curve | 11.74                   | 0.694          | 106.75         | acid water | 76.3                |
| Control        | 12.67                   | 0.694          | 115.24         | acid water | 76.3                |

\* = Molecular weights of pyridostigmine cation/pyridostigmine hydrochloride

\*\* = Water acidified by addition of a drop of 1 N HCl.

##### b. Neostigmine bromide expressed as the bromide concentration-Internal standard for interday plasma precision.

Prep date: 11/14/94

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent  | Conc. ( $\mu$ g/ml) |
|---------------|-------------------------|---------------|----------------|----------|---------------------|
| Internal std. | 7.79                    | 1             | 103.9          | 50% MeOH | 75.0                |

6. REPRESENTATIVE WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and discarded when stock solutions were discarded or by 6 months after the preparation date).

a. Pyridostigmine (cation) solutions.

Prep date: 11/23/94

| Solution Type  | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent*   | Conc.<br>( $\mu\text{g}/\text{ml}$ ) |
|----------------|-------------------------------------------------|---------------------------|----------------------|------------|--------------------------------------|
| Standard Curve | 76.3                                            | 0.500                     | 50                   | acid water | 0.763                                |
| Control        | 76.3                                            | 0.500                     | 50                   | acid water | 0.763                                |

\*\* = Water acidified by addition of a drop of 1 N HCl.

b. Neostigmine bromide - Internal standard for interday plasma precision.

Prep date: 9/7/94

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent  | Conc.<br>( $\mu\text{g}/\text{ml}$ ) |
|---------------|-------------------------------------------------|---------------------------|----------------------|----------|--------------------------------------|
| Internal std. | 75.0                                            | 1                         | 200                  | 50% MeOH | 0.375                                |

7. RETENTION TIMES (subject to change depending on temperature and column performance).

- a. Neostigmine bromide (Internal Standard) - 14 min  
b. Pyridostigmine (cation) - 16 min

8. BLANK PLASMA AND BLOOD

Human plasma (CPD or CPDA-1 as anticoagulant) is obtained from the San Francisco Irwin Memorial Blood Bank.

9. INJECTION VOLUME

25-50  $\mu\text{l}$  - Samples that are expected to have high pyridostigmine concentrations (i.e. high standard curve calibrators, high concentration control samples, and sponsor samples shown in early runs to be near  $C_{\text{peak}}$  or in later runs expected to be near  $C_{\text{peak}}$ ) are injected at the low end of the volume range.

10. BOND ELUT CARTRIDGES

C8 Bond Elut (500 mg packing, Varian, Harbor City, CA). New C8 Bond Elut cartridges were prepared by washing (fill up the columns) with  $\text{CH}_3\text{CN}$  and water.

## 11. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves are calculated by weighted linear regression where weights =  $1/y$ .

## 12. LOWER LIMIT OF QUANTITATION OF METHOD (The minimum pyridostigmine (cation) quantitation limit for the assay of human plasma was based on the interday and intraday low point validation results, on standard curve calibrator results, and a minimum 3 to 1 signal to noise ratio.)

1.53 ng/ml pyridostigmine (cation) in plasma.

## 13. VOLUME MEASUREMENT

Plasma sample volumes were measured with a 200  $\mu$ l or a 1000  $\mu$ l Gilson Pipetman. Blood sample volumes were measured with Eppendorf pipettes. Hamilton syringes were used to measure standard and control solution volumes.

## 14. WISP OPERATING TEMPERATURE

Room temperature.

## 15. SATURATING THE MOBILE PHASE WITH SILICA

The mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica.

## 16. SAMPLE EVAPORATION

Extracted samples are evaporated in a N-EVAP® Model 112 (Organomatic Assoc, Inc., S.Berlin, MA) by passing  $N_2$  over the sample. The samples do not sit in water during evaporation.

## D. SAMPLE STORAGE

All samples were kept frozen at  $-70^{\circ}\text{C}$  before analysis and thawed for preparation and analysis, unless specified otherwise.

## E. SAMPLE PREPARATION

1. Vortex frozen specimens for 20 seconds after sample thaws.
2. Pipet 0.5 ml of a plasma sample into a clean glass culture tube (13 x 100).

3. Spike standard curve samples as shown in Section G "Generation of Standard Curve Calibrators" and vortex for 10 s.
4. Add 50  $\mu$ l of internal standard (0.375  $\mu$ g/ml neostigmine bromide) solution.
5. Vortex for 10 seconds.
6. Add 1 ml  $\text{CH}_3\text{CN}$  to precipitate plasma proteins.
7. Vortex 1 min.
8. Centrifuge 10 minutes at 3000 g.
9. Pour supernatant into a prewashed C8 Bond Elut cartridge (500 mg) and successively wash with 2 ml  $\text{H}_2\text{O}$ , 4 ml  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (50:50), 2 ml  $\text{CH}_3\text{CN}$ , and 0.5 ml  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (85:15) containing 1.0 mM  $(\text{NH}_4)_2\text{HPO}_4$  at pH 3.6. Discard washings. Elutions from Bond Elut cartridges were performed using the gravity drip method.
10. Elute with 2 ml  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (85:15) containing 1.0 mM  $(\text{NH}_4)_2\text{HPO}_4$  at pH 3.6.
11. Evaporate to approximately 200  $\mu$ l under  $\text{N}_2$  at room temperature.
12. Transfer to WISP vial and inject onto HPLC column.

#### F. QUALITY CONTROL

1. Content and frequency of blanks

A blank plasma sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

2. PIPETTE CALIBRATION

See SOP 2C-1.1.

3. BALANCE CALIBRATION

See SOP 2C-2.1

#### G. GENERATION OF STANDARD CURVE CALIBRATORS

A representative example of the generation of standard curve calibrators is shown in the table below. Spike blank plasma standard curve samples with pyridostigmine (cation) solution to make a standard curve. This procedure is equivalent to addition of the

masses of pyridostigmine (cation) shown below. Since 0.500 ml plasma samples are assayed, these amounts correspond to the nominal cation concentrations shown below. Vortex for 10s.

Generation of Pyridostigmine (cation)  
Standard Curve Samples

| Sample | Volume Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Mass Spiked<br>(ng) | Standard Curve Sample<br>Nominal Concentration<br>(ng/ml) |
|--------|-----------------------------|----------------------------------------------------|---------------------|-----------------------------------------------------------|
| 00*    | 0                           | 0                                                  | 0                   | 0                                                         |
| 0**    | 0                           | 0                                                  | 0                   | 0                                                         |
| 1      | 1                           | 0.763                                              | 0.763               | 1.53                                                      |
| 2      | 2                           | 0.763                                              | 1.526               | 3.05                                                      |
| 3      | 4                           | 0.763                                              | 3.052               | 6.10                                                      |
| 4      | 8                           | 0.763                                              | 6.104               | 12.2                                                      |
| 5      | 15                          | 0.763                                              | 11.45               | 22.9                                                      |
| 6      | 25                          | 0.763                                              | 19.08               | 38.2                                                      |
| 7      | 40                          | 0.763                                              | 30.52               | 61.0                                                      |
| 8      | 50                          | 0.763                                              | 38.15               | 76.3                                                      |

#### H. GENERATION OF PRECISION SAMPLES

A representative example of the generation of precision controls is shown in the table below. Samples for precision analysis were prepared by spiking 0.5 ml plasma specimens with control working solutions to make the pyridostigmine (cation) concentrations shown.

Generation of Pyridostigmine Precision Samples

|      | Volume Spiked<br>( $\mu$ l) | Spiking Solution<br>Concentration<br>( $\mu$ g/ml) | Control<br>Volume<br>(ml) | Precision Sample<br>Nominal Concentration<br>(ng/ml) |
|------|-----------------------------|----------------------------------------------------|---------------------------|------------------------------------------------------|
| X-Lo | 2                           | 0.763                                              | 0.5                       | 3.05                                                 |
| Low  | 6                           | 0.763                                              | 0.5                       | 9.16                                                 |
| Med. | 20                          | 0.763                                              | 0.5                       | 30.5                                                 |
| Hi   | 30                          | 0.763                                              | 0.5                       | 45.8                                                 |

#### I. GENERATION OF RECOVERY SAMPLES

Assay recovery was assessed at four different concentrations by comparing the pyridostigmine (cation) to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.5 ml) samples were spiked with pyridostigmine (cation) then prepared as described above in "Sample Preparation," except the internal standard was added after the elution (step 10). Reference samples were generated by preparing 0.5 ml plasma as described above in "Sample Preparation," except that pyridostigmine was spiked and internal standard added after the elution.

\*00 = Sample with no drug and no internal standard.

\*\*0 = Sample with no drug but with internal standard.

## J. GENERATION OF STABILITY SAMPLES

System stability and bench top stability samples were generated in the same way as precision control samples.

The effect of repeated freeze and thaw cycles on stabilities of pyridostigmine (cation) in human plasma samples was determined as follows: Spiked (low and high concentrations) pooled biological samples were subjected to five thaw/freeze cycles. For each cycle, a duplicate set of thaw/freeze samples (0.5 ml) was generated at each concentration. The study is run with the following procedure:

- a. Prepare high and low concentration samples labeled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.
- b. Store all samples until frozen at the specified temperature.
- c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 h.

| Cycle | Keep these<br>samples in freezer | Thaw these<br>samples |
|-------|----------------------------------|-----------------------|
| 1     | 1                                | 2, 3, 4, 5            |
| 2     | 1, 2                             | 3, 4, 5               |
| 3     | 1, 2, 3                          | 4, 5                  |
| 4     | 1, 2, 3, 4                       | 5                     |
| 5     | 1, 2, 3, 4, 5                    | none                  |

- d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

## K. VALIDATION RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in representative standard curves for pyridostigmine (cation) appear in Figure 3. Peak height ratios for these calibrators appear in Table 1. Statistical parameters of plasma interday precision standard curve calibrators appear in Table 2.

### 2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Tables 3A-B.

3. LLOQ

Results for this evaluation appear in Table 4.

4. RECOVERY

Results for this evaluation appear in Table 5.

5. STABILITY

a. System Stability: Results appear in Table 6.

b. Long Term Stability: Results appear in Table 7.

c. Bench Top Stability: Results appear in Table 8.

d. Freeze/Thaw Stability: Results appear in Table 9.

6. BLIND SAMPLE ANALYSIS

Results appear in Table 10.

TABLE 1: REPRESENTATIVE STANDARD CURVE FOR  
PYRIDOSTIGMINE (FREE BASE)  
HUMAN PLASMA ASSAY,  
STUDY REPORT 25

| SPIKED<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE        |       | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|---------------------------|--------------------------|-------|---------------------------|----------------------------------------|
|                           | CONCENTRATION<br>(ng/ml) |       |                           |                                        |
| 0                         | 0                        | -     | -                         | -                                      |
| 0.7630                    | 1.53                     | 0.061 | 0.061                     | 1.41                                   |
| 1.526                     | 3.05                     | 0.145 | 0.145                     | 3.36                                   |
| 3.052                     | 6.10                     | 0.259 | 0.259                     | 6.01                                   |
| 6.104                     | 12.2                     | 0.527 | 0.527                     | 12.2                                   |
| 11.45                     | 22.9                     | 1.022 | 1.022                     | 23.7                                   |
| 19.08                     | 38.2                     | 1.569 | 1.569                     | 36.4                                   |
| 30.52                     | 61.0                     | 2.699 | 2.699                     | 62.6                                   |
| 38.15                     | 76.3                     | 3.268 | 3.268                     | 75.8                                   |

Regression equation:\*\*\*

$$y = 0.0431x + 0.00002, \quad r^2 = 0.9988$$

\* Into 0.5 ml of biological sample.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where  
weight =  $1/y_i$ .

Representative Standard Curve

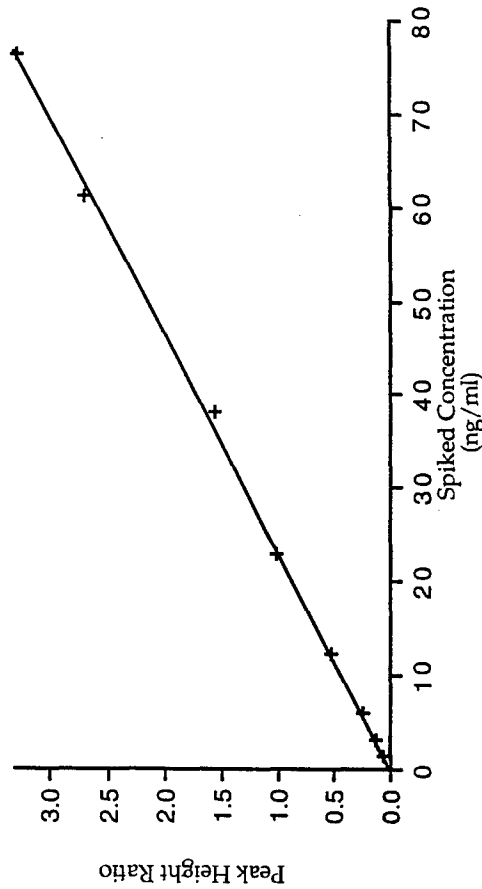


**TABLE 2: PRECISION STANDARD CURVE DATA FOR PYRIDOSTIGMINE  
HUMAN PLASMA ASSAY, SR 25**

Pyridostigmine Cation Standard Curve Parameters

| Validation Run<br>Date | Validation Run<br>No. | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|-----------------------|------------|------------|---------------------------------|
| 11/28/94               | 1                     | 0.03895819 | 0.02074876 | 0.99767455                      |
| 12/2/94                | 2                     | 0.03970787 | 0.01593752 | 0.99814691                      |
| 12/3/94                | 3                     | 0.0431179  | 2.3361E-05 | 0.9988433                       |
| 12/2/94                | 4                     | 0.04117508 | -0.0084501 | 0.99885733                      |
| 12/4/94                | 5                     | 0.04069309 | 0.0099219  | 0.99693227                      |
| 12/6/94                | 6                     | 0.04602067 | 0.0217952  | 0.9960465                       |
| 12/20/94               | 7                     | 0.04662032 | 0.00526143 | 0.99715104                      |

Pyridostigmine Cation Back Calculated Standard Calibrators

| Run<br>Number     | Spiked Concentration (ng/mL)          |       |       |       |       |       |       |      |
|-------------------|---------------------------------------|-------|-------|-------|-------|-------|-------|------|
|                   | 1.53                                  | 3.05  | 6.1   | 12.2  | 22.9  | 38.2  | 61    | 76.3 |
|                   | Back Calculated Concentration (ng/mL) |       |       |       |       |       |       |      |
| 1                 | 1.24                                  | 3.63  | 5.94  | 12.9  | 24.2  | 35.9  | 61.9  | 76.0 |
| 2                 | 1.69                                  | 3.00  | 6.30  | 11.4  | 21.7  | 38.2  | 62.9  | bc   |
| 3                 | 1.41                                  | 3.36  | 6.01  | 12.2  | 23.7  | 36.4  | 62.6  | 75.8 |
| 4                 | 1.37                                  | 3.17  | 6.50  | 13.3  | 21.9  | 38.0  | 60.4  | 76.7 |
| 5                 | 1.60                                  | 3.47  | 5.78  | 12.1  | 20.8  | 36.3  | 62.6  | 79.1 |
| 6                 | 1.29                                  | bc    | 6.35  | 13.1  | 24.3  | 40.1  | 56.4  | 77.3 |
| 7                 | 1.47                                  | 3.58  | 6.15  | 11.7  | 21.5  | 35.8  | 62.9  | 78.7 |
| n                 | 7                                     | 6     | 7     | 7     | 7     | 7     | 7     | 6    |
| Mean              | 1.44                                  | 3.37  | 6.15  | 12.4  | 22.6  | 37.2  | 61.4  | 77.3 |
| S.D.              | 0.162                                 | 0.244 | 0.253 | 0.727 | 1.44  | 1.59  | 2.37  | 1.38 |
| Percent CV        | 11.3                                  | 7.26  | 4.12  | 5.87  | 6.37  | 4.26  | 3.86  | 1.78 |
| Percent Rel. Err. | -5.98                                 | 10.4  | 0.773 | 1.52  | -1.37 | -2.51 | 0.632 | 1.27 |

bc = unacceptable chromatogram

**TABLE 3: PRECISION OF PYRIDOSTIGMINE (CATION) HUMAN PLASMA ASSAY**

Inter-Run Precision Pyridostigmine Cation

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |      |       |        |
|---------------------------------|------------------|-------------------------------|------|-------|--------|
|                                 |                  | 3.05                          | 9.16 | 30.5  | 45.8   |
| Measured Concentrations (ng/mL) |                  |                               |      |       |        |
| 1                               | 1                | 3.73                          | 9.86 | 30.7  | 51.3   |
|                                 | 2                | 3.34                          | 9.79 | 31.8  | 51.4   |
| 2                               | 1                | 3.10                          | 11.7 | 30.4  | 41.3   |
|                                 | 2                | 2.90                          | 12.3 | 29.4  | 47.0   |
| 3                               | 1                | 3.22                          | 8.16 | 31.8  | 48.9   |
|                                 | 2                | 3.34                          | 9.49 | 30.3  | 47.0   |
| 4                               | 1                | 3.61                          | 9.17 | 29.4  | 33.3   |
|                                 | 2                | 2.76                          | 7.32 | 30.0  | 41.6   |
| 5                               | 1                | 3.27                          | 9.71 | 29.5  | 49.7   |
|                                 | 2                | bc                            | 9.09 | 29.6  | 47.7   |
| 6                               | 1                | 2.66                          | 6.54 | 32.1  | 46.6   |
|                                 | 2                | 2.81                          | 9.54 | 31.0  | 43.2   |
| n                               |                  | 11                            | 12   | 12    | 12     |
| Mean                            |                  | 3.16                          | 9.39 | 30.5  | 45.8   |
| S.D.                            |                  | 0.348                         | 1.61 | 0.989 | 5.17   |
| Percent CV                      |                  | 11.0                          | 17.1 | 3.24  | 11.3   |
| Percent R.E.                    |                  | 3.55                          | 2.50 | 0     | -0.109 |

Intra-Run Precision Pyridostigmine Cation

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |       |       |
|---------------------------------|------------------|-------------------------------|-------|-------|-------|
|                                 |                  | 3.05                          | 9.16  | 30.5  | 45.8  |
| Measured Concentrations (ng/mL) |                  |                               |       |       |       |
| 7                               | 1                | 2.80                          | 9.15  | 28.7  | 39.5  |
|                                 | 2                | 2.53                          | 8.85  | 29.1  | 41.6  |
|                                 | 3                | 3.21                          | 8.51  | 29.7  | 42.8  |
|                                 | 4                | 3.25                          | 8.92  | 28.9  | 42.9  |
|                                 | 5                | 3.25                          | 9.48  | 29.6  | 46.5  |
|                                 | 6                | 3.00                          | 9.50  | 31.2  | 44.5  |
| Mean                            |                  | 3.01                          | 9.07  | 29.5  | 43.0  |
| S.D.                            |                  | 0.293                         | 0.386 | 0.905 | 2.398 |
| Percent CV                      |                  | 9.75                          | 4.25  | 3.06  | 5.58  |
| Percent R.E.                    |                  | -1.42                         | -1.00 | -3.17 | -6.19 |

**TABLE 4: LOWER LIMIT OF QUANTITATION OF THE HUMAN PLASMA ASSAY FOR PYRIDOSTIGMINE BASE**

| Spiked Concentration | 1.53 ng/ml                         | 1.53 ng/ml |
|----------------------|------------------------------------|------------|
| Sample               | Measured Concentrations<br>(ng/ml) |            |
|                      | Interday                           | Intraday   |
| 1                    | 1.24                               | 1.88       |
| 2                    | 1.69                               | 1.55       |
| 3                    | 1.41                               | 1.57       |
| 4                    | 1.37                               | 1.34       |
| 5                    | 1.60                               | 1.96       |
| 6                    | 1.29                               | 1.50       |
| Mean                 | 1.43                               | 1.63       |
| Standard Deviation   | 0.18                               | 0.24       |
| Percent CV           | 12.3                               | 14.6       |
| Percent Error        | -6.32                              | 6.72       |

**TABLE 5: RECOVERY OF PYRIDOSTIGMINE FROM HUMAN PLASMA**

| SAMPLE ID        | SPIKED CONCENTRATION<br>Range (ng/ml) | PEAK HEIGHT RATIO |                   | MEAN PERCENT RECOVERY |
|------------------|---------------------------------------|-------------------|-------------------|-----------------------|
|                  |                                       | SOLVENT           | PLASMA            |                       |
| 1                | X Low                                 | 0.137             | 0.080             | 62.7                  |
| 2                |                                       | 0.121             | 0.080             |                       |
| 3                |                                       | 0.131             | 0.084             |                       |
| Mean ( $\pm$ SD) | 3.05                                  | 0.130 $\pm$ 0.008 | 0.081 $\pm$ 0.002 |                       |
| 1                | Low                                   | 0.355             | 0.239             | 67.6                  |
| 2                |                                       | 0.331             | 0.225             |                       |
| 3                |                                       | 0.344             | 0.232             |                       |
| Mean ( $\pm$ SD) | 9.16                                  | 0.343 $\pm$ 0.012 | 0.232 $\pm$ 0.007 |                       |
| 1                | Medium                                | 1.096             | 0.763             | 69.1                  |
| 2                |                                       | 1.126             | 0.770             |                       |
| 3                |                                       | 1.102             | 0.764             |                       |
| Mean ( $\pm$ SD) | 30.5                                  | 1.108 $\pm$ 0.016 | 0.766 $\pm$ 0.004 |                       |
| 1                | High                                  | 1.717             | 1.175             | 67.9                  |
| 2                |                                       | 1.682             | 1.156             |                       |
| 3                |                                       | 1.695             | 1.127             |                       |
| Mean ( $\pm$ SD) | 45.8                                  | 1.698 $\pm$ 0.018 | 1.153 $\pm$ 0.024 |                       |
| AVERAGE =        |                                       |                   |                   | 66.8                  |

**TABLE 6: SYSTEM STABILITY IN PREPARED HUMAN PLASMA****Concentration for Prepared Biological Samples Stored at Room Temperature****Pyridostigmine (Cation)**

|                       |  | CONCENTRATION* |      |      |      |
|-----------------------|--|----------------|------|------|------|
|                       |  | (ng/ml)        |      |      |      |
| Spiked Concentration: |  | 3.05           | 9.16 | 30.5 | 45.8 |
| TIME STORED           |  |                |      |      |      |
| 0 day                 |  | 2.74           | 8.04 | 31.6 | 44.9 |
| 1 day                 |  | 2.85           | 8.79 | 29.6 | 46.9 |
| 2 day                 |  | 2.28           | 9.00 | 29.8 | 43.9 |
| 3 day                 |  | 2.94           | 8.37 | 30.2 | 44.0 |
| 4 day                 |  | 2.58           | 8.85 | 29.8 | 45.5 |
| 5 day                 |  | 2.47           | 9.02 | 30.0 | 45.8 |
| 6 day                 |  | 2.55           | 8.75 | 29.7 | 45.5 |

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\* Measured concentrations are averages of two analyses.

**TABLE 7: FREEZER STABILITY OF PYRIDOSTIGMINE (CATION) IN HUMAN PLASMA<sup>#</sup>**

**PYRIDOSTIGMINE (CATION) CONCENTRATION IN PLASMA STORED AT -80°C**

|                       |  | CONCENTRATION* (ng/ml) |                 |                 |                 |
|-----------------------|--|------------------------|-----------------|-----------------|-----------------|
| Spiked Concentration: |  | 3.43                   | 10.3            | 27.3            | 47.8            |
| DAYS STORED           |  |                        |                 |                 |                 |
| 0                     |  | 4.25                   | 11.1            | 26.6            | 43.5            |
| 1                     |  | 3.16                   | 10.4            | 25.8            | 46.0            |
| 2                     |  | 3.57                   | 10.4            | 24.4            | 46.6            |
| 3                     |  | 3.70                   | 10.2            | 26.6            | 47.8            |
| 6                     |  | 2.81                   | 9.60            | 26.9            | 29.6            |
| 13                    |  | 3.77                   | 9.88            | 24.5            | 44.9            |
| 20                    |  | 3.50                   | 10.4            | 24.8            | 44.3            |
| 29                    |  | 3.16                   | 10.0            | 28.1            | 48.4            |
| 57                    |  | 4.08                   | 12.6            | 29.8            | 49.3            |
| 99                    |  | 2.54                   | 8.64            | 26.2            | 43.8            |
| 135                   |  | 4.21                   | 10.8            | 29.3            | 45.3            |
| MEAN $\pm$ SD         |  | 3.52 $\pm$ 0.56        | 10.4 $\pm$ 0.98 | 26.6 $\pm$ 1.82 | 46.3 $\pm$ 2.18 |

**PYRIDOSTIGMINE (CATION) CONCENTRATION IN PLASMA STORED AT -20°C**

|                       |  | CONCENTRATION (ng/ml) |      |      |      |
|-----------------------|--|-----------------------|------|------|------|
| Spiked Concentration: |  | 3.43                  | 10.3 | 27.3 | 47.8 |
| DAYS STORED           |  |                       |      |      |      |
| 0                     |  | 3.16                  | 9.47 | 22.4 | 42.2 |
| 1                     |  | 3.16                  | 9.75 | 28.1 | 47.2 |
| 3                     |  | 3.50                  | 9.95 | 28.9 | 44.3 |
| 6                     |  | 4.66                  | 9.95 | 28.8 | 42.9 |
| 13                    |  | 2.61                  | 10.1 | 25.2 | 37.5 |
| 20                    |  | 3.16                  | 9.19 | 23.1 | 35.7 |
| 57                    |  | 2.32                  | 6.72 | 18.7 | 39.7 |
| 99                    |  | 0.75                  | 4.32 | 6.79 | 16.2 |
| 135                   |  | 1.87                  | 3.90 | 6.08 | 15.8 |

<sup>#</sup> Data obtained according to the method described in Study Report No. 3, dated Jan. 22, 1985 and titled "High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma."

\* Measured concentrations are averages of two analyses.

**TABLE 8: BENCH TOP STABILITY OF PYRIDOSTIGMINE (CATION) IN SPIKED HUMAN PLASMA #**

**CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE**

|                       |  | CONCENTRATION<br>(ng/ml) |      |      |      |
|-----------------------|--|--------------------------|------|------|------|
| Spiked Concentration: |  | 2.86                     | 9.54 | 30.5 | 45.8 |
| TIME STORED           |  |                          |      |      |      |
| 0 hour                |  | 2.88                     | 9.92 | 30.4 | 46.5 |
| 1 hour                |  | 2.91                     | 9.53 | 30.9 | 50.4 |
| 2 hour                |  | 2.73                     | 9.80 | 28.4 | 43.5 |
| 4 hour                |  | 2.37                     | 8.33 | 27.0 | 43.3 |
| 6 hour                |  | 2.79                     | 8.71 | 25.5 | 42.6 |

**TABLE 9: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON PYRIDOSTIGMINE (CATION) SPIKED HUMAN PLASMA SAMPLES@ #**

| Spiked<br>Concentration | AT ROOM TEMPERATURE  |                       | ON ICE               |                       |
|-------------------------|----------------------|-----------------------|----------------------|-----------------------|
|                         | Low<br>Concentration | High<br>Concentration | Low<br>Concentration | High<br>Concentration |
|                         | (9.54 ng/ml)         | (45.8 ng/ml)          | (9.54 ng/ml)         | (45.8 ng/ml)          |
| Cycle                   |                      |                       |                      |                       |
| 1                       | 9.41                 | 46.7                  | 8.08                 | 43.8                  |
| 2                       | 8.52                 | 44.3                  | 8.13                 | 41.7                  |
| 3                       | 8.05                 | 41.8                  | 8.28                 | 40.5                  |
| 4                       | 8.54                 | 45.2                  | 8.50                 | 40.0                  |
| 5                       | 8.08                 | 39.9                  | 8.49                 | 43.9                  |

# Measured concentrations are averages of two analyses.

@ Individually spiked samples.

**TABLE 10: ACCURACY OF PYRIDOSTIGMINE (CATION) HUMAN PLASMA ASSAY (BLIND STUDY RESULTS)**

| Sample Number | Spiked Level (ng/ml) | Measured Level <sup>#</sup> <sup>¥</sup> (ng/ml) | Statistics (ng/ml)   |
|---------------|----------------------|--------------------------------------------------|----------------------|
| 3             | 0                    | *                                                | Mean =               |
| 13            |                      | *                                                | SD =                 |
| 18            |                      | *                                                | Percent CV =         |
| 28            |                      | *                                                | Percent Bias =       |
| 6             | 2.71                 | 2.74                                             | Mean = 3.04          |
| 10            |                      | 3.27                                             | SD = 0.42            |
| 15            |                      | 2.87                                             | Percent CV = 13.78   |
| 20            |                      | 2.35                                             | Percent Bias = 12.18 |
| 24            |                      | 3.15                                             |                      |
| 27            |                      | 3.61                                             |                      |
| 29            |                      | 3.30                                             |                      |
| 1             | 8.12                 | 9.48                                             | Mean = 8.78          |
| 4             |                      | 5.46                                             | SD = 1.59            |
| 8             |                      | 8.23                                             | Percent CV = 18.11   |
| 17            |                      | 9.08                                             | Percent Bias = 8.13  |
| 26            |                      | 9.42                                             |                      |
| 30            |                      | 9.52                                             |                      |
| 32            |                      | 10.3                                             |                      |
| 5             | 18.97                | 18.4                                             | Mean = 20.1          |
| 12            |                      | 21.9                                             | SD = 2.51            |
| 14            |                      | 19.6                                             | Percent CV = 12.47   |
| 21            |                      | 18.4                                             | Percent Bias = 5.96  |
| 22            |                      | 19.2                                             |                      |
| 25            |                      | 18.2                                             |                      |
| 31            |                      | 25.0                                             |                      |
| 2             | 24.33                | 23.3                                             | Mean = 25.10         |
| 7             |                      | 26.7                                             | SD = 1.52            |
| 9             |                      | 23.3                                             | Percent CV = 6.06    |
| 11            |                      | 25.0                                             | Percent Bias = 3.16  |
| 16            |                      | 27.1                                             |                      |
| 19            |                      | 24.5                                             |                      |
| 23            |                      | 25.8                                             |                      |

<sup>#</sup> Measured concentrations are averages of three analyses.

<sup>¥</sup> Data obtained according to the method described in Study Report No. 5, dated July 21, 1986 and titled "High Pressure Liquid Chromatography (HPLC) of Pyridostigmine in Plasma Using Silica Gel Column and an Aqueous Mobile Phase."

\* = Below assay sensitivity.

**LABORATORY METHODOLOGY FOR WR 242511 HUMAN AND DOG  
PLASMA ASSAY,\* STUDY REPORT 26**

**A. INSTRUMENTS**

1. Waters Intelligent Sample Processor Model 717 (Waters Associates, Milford, MA) or equivalent.
2. LC-600 Shimadzu Pump (Shimadzu Corp., Kyoto, Japan) or equivalent.
3. Shimadzu SPD 10A UV Detector (Shimadzu Corp., Kyoto, Japan) or equivalent.
4. Hewlett-Packard Reporting Integrator #3392A (Hewlett-Packard Co., Santa Clara, CA) or equivalent.

**B. REAGENTS**

1. WR 242511, bottle no. BM 19356 (WRAIR, Washington D.C.).
2. Chlorpheniramine (Internal Standard).
3. Triethylamine (TEA), HPLC grade, (Aldrich Chemical Co., Milwaukee, WI).
4. Acetonitrile, HPLC grade, (Fisher Scientific, Fair Lawn, NJ).
5. Phosphoric acid, reagent grade, (Fisher Scientific, Fair Lawn, NJ).
6. Sodium hydroxide, reagent grade, (Fisher Scientific, Fair Lawn, NJ).
7. Methyl *t*-butyl ether (omnisolv distilled) (EM Science, Biggstown, NJ).
8. Water, Type 1 reagent grade, (deionized by Nanopure II, Barnstead Co., Boston, MA).

\*Minor alterations may have been made in the assay process, depending on instrument and assay conditions.

### C. ASSAY CONDITIONS

#### 1. DETECTOR

##### Settings

Wavelength; 240 nm

Range: 0.003 aufs

##### Lamp

Deuterium Lamp, ABI Analytical, Inc., Ramsey, NJ.

#### 2. COLUMN

Axxiom Silica, 5  $\mu$ m particle size, 4.6 x 250 mm (Richard Scientific, Novato, CA) or equivalent.

#### 3. SOLVENT SYSTEM

CH<sub>3</sub>CN/H<sub>2</sub>O (7:3, v/v) with 0.008% TEA and 0.005% H<sub>3</sub>PO<sub>4</sub>  
(final concentrations)

#### 4. FLOW RATE

1.0 ml/min

#### 5. STOCK SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and checked for deterioration by comparison to a newly made solution (solutions are discarded when a more than 10% change in the absolute peak height is observed or by 2 months after the preparation date).

- a. WR 242511 tartrate for interday human plasma precision expressed as the free base concentration.

Prep date: 9/21/95

| Solution Type  | Weight of Standard (mg) | Purity Factor* | QS Volume (ml) | Solvent                | Conc. ( $\mu$ g/ml) |
|----------------|-------------------------|----------------|----------------|------------------------|---------------------|
| Standard Curve | 14.13                   | 0.714          | 50.44          | 50% CH <sub>3</sub> CN | 200                 |
| Control        | 14.10                   | 0.714          | 50.33          | 50% CH <sub>3</sub> CN | 200                 |

\*= Molecular weights of WR 242511 free base/WR 242511 (as DL tartrate)

- b. Chlorpheniramine maleate expressed as the maleate concentration- Internal standard for interday human plasma precision.

Prep date: 9/21/95

| Solution Type | Weight of Standard (mg) | Purity Factor | QS Volume (ml) | Solvent                | Conc. ( $\mu$ g/ml) |
|---------------|-------------------------|---------------|----------------|------------------------|---------------------|
| Internal std. | 10.45                   | 1             | 104.5          | 50% CH <sub>3</sub> CN | 100                 |

6. WORKING SOLUTIONS - Solutions were stored in a 4°C refrigerator, protected from light in an amber bottle (or wrapped in aluminum foil), and discarded when stock solutions were discarded or by 6 months after the preparation date).

- a. WR 242511 tartrate solutions expressed as the free base concentration.

| Solution Type  | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent                    | Conc.<br>( $\mu\text{g}/\text{ml}$ ) |
|----------------|-------------------------------------------------|---------------------------|----------------------|----------------------------|--------------------------------------|
| Standard Curve | 200                                             | 6.40                      | 10                   | 50% $\text{CH}_3\text{CN}$ | 128                                  |
| Control        | 200                                             | 6.40                      | 50                   | 50% $\text{CH}_3\text{CN}$ | 128                                  |

- b. Chlorpheniramine maleate - Internal standard.

| Solution Type | Conc.<br>Diluted<br>( $\mu\text{g}/\text{ml}$ ) | Volume<br>Diluted<br>(ml) | QS<br>Volume<br>(ml) | Solvent                    | Conc.<br>( $\mu\text{g}/\text{ml}$ ) |
|---------------|-------------------------------------------------|---------------------------|----------------------|----------------------------|--------------------------------------|
| Internal std. | 100                                             | 1.00                      | 50                   | 50% $\text{CH}_3\text{CN}$ | 2.00                                 |

7. RETENTION TIMES (subject to change depending on temperature and column performance).

- a. WR 242511 - 10.5 min  
b. Chlorpheniramine (Internal Standard) - 15.5 min

#### 8. BLANK PLASMA

Human plasma (CPD or CPDA-1 as anticoagulant) was obtained from the San Francisco Irwin Memorial Blood Bank. Dog plasma (EDTA as anticoagulant) was obtained from Pel-Freez Biologicals, Rogers, AK.

#### 9. INJECTION VOLUME

40  $\mu\text{l}$

#### 10. QUANTITATION

By peak height ratio of drug peak relative to internal standard peak. Standard curves are calculated by weighted linear regression where weights =  $1/y$ .

11. LOWER LIMIT OF QUANTITATION OF METHOD (The lower limit of quantitation of the assay of human and dog plasma for WR 242511 was based on the interday and intraday low point validation results, on standard curve calibrator results, and a minimum 3 to 1 signal to noise ratio.)

4.00 ng/ml WR 242511 in plasma.

12. VOLUME MEASUREMENT

Plasma sample volumes were measured with a 200  $\mu$ l or a 1000  $\mu$ l Gilson Pipetman. Hamilton syringes were used to measure standard and control solution volumes.

13. WISP OPERATING TEMPERATURE

Room temperature.

14. SATURATING THE MOBILE PHASE WITH SILICA

The mobile phase is recycled through a non analytical silica gel column overnight to saturate it with silica prior to use.

#### D. SAMPLE STORAGE

All samples were kept frozen at -70°C before analysis and thawed for preparation and analysis, unless specified otherwise.

#### E. SAMPLE PREPARATION

1. If frozen, vortex specimens for 20 seconds after sample thaws.
2. Pipet 0.5 ml of a plasma sample into a clean glass culture tube.
3. Spike standard curve sample then perform serial dilution as shown in Section G "Generation of Standard Curve Calibrators."
4. Add 0.5 ml of internal standard (2  $\mu$ g/ml chlorpheniramine) solution. Vortex for 10 seconds.
5. Add 100  $\mu$ l of 0.1N NaOH. Vortex for 10 seconds.
6. Add 3 ml methyl *t*-butyl ether. Vortex 1 min, twice.
7. Centrifuge 10 minutes at 3000 *g*.
8. Freeze in dry ice/methanol bath.

9. Pour supernatant into a clean glass culture tube and evaporate under  $N_2$  at room temperature to dryness. Reconstitute in 200  $\mu$ l of 70% acetonitrile.
10. Transfer to WISP vial and inject 40  $\mu$ l onto HPLC column.

#### F. QUALITY CONTROL

1. Content and frequency of blanks

A blank plasma sample was prepared as described in "Sample Preparation" and assayed at least once for each standard curve in precision assays.

2. PIPETTE CALIBRATION

See SOP 2C-1.2.

3. BALANCE CALIBRATION

See SOP 2C-2.1

#### G. GENERATION OF STANDARD CURVE CALIBRATORS

The generation of standard curve calibrators is described in the table below. Standard curve samples (0.5 ml) were generated by serial dilution of a 1024 ng/ml plasma sample (spiked with 40  $\mu$ l of 128  $\mu$ g/ml WR 242511 working solution, q.s. with plasma to 5 ml and vortexed 1 min).

Generation of WR 242511 Standard Curve Calibrators

| Sample Number | Concentration in Plasma Diluted (ng/ml) | Volume Taken for Dilution (ml) | Volume of Blank Plasma Added (ml) | Standard Curve Sample Concentration (ng/ml) |
|---------------|-----------------------------------------|--------------------------------|-----------------------------------|---------------------------------------------|
| 9             | -                                       | -                              | -                                 | 1024                                        |
| 8             | 1024                                    | 1.00                           | 1.00                              | 512                                         |
| 7             | 512                                     | 1.00                           | 1.00                              | 256                                         |
| 6             | 256                                     | 1.00                           | 1.00                              | 128                                         |
| 5             | 128                                     | 1.00                           | 1.00                              | 64.0                                        |
| 4             | 64.0                                    | 1.00                           | 1.00                              | 32.0                                        |
| 3             | 32.0                                    | 1.00                           | 1.00                              | 16.0                                        |
| 2             | 16.0                                    | 1.00                           | 1.00                              | 8.00                                        |
| 1             | 8.00                                    | 1.00                           | 1.00                              | 4.00                                        |
| 00*           | -                                       | -                              | -                                 | 0                                           |
| 0**           | -                                       | -                              | -                                 | 0                                           |

\*00 = Sample with no drug and no internal standard.

\*\*0 = Sample with no drug but with internal standard.

## H. GENERATION OF PRECISION SAMPLES

Interday precision samples (0.5 ml) were generated by serial dilution of a 256 ng/ml plasma sample (spiked with 10  $\mu$ l of 128  $\mu$ g/ml WR 242511 working solution, q.s. with plasma to 5 ml and vortexed 1 min). For intraday precision, volumes (spiking solution, q.s. plasma, taken plasma and blank plasma) were doubled.

### Generation of WR 242511 Interday Precision Samples

| Sample Number | Concentration in Plasma Diluted (ng/ml) | Volume Taken for Dilution (ml) | Volume of Blank Plasma Added (ml) | Standard Curve Sample Concentration (ng/ml) |
|---------------|-----------------------------------------|--------------------------------|-----------------------------------|---------------------------------------------|
| Hi            | -                                       | -                              | -                                 | 256                                         |
| Med.          | 256                                     | 2.00                           | 2.00                              | 128                                         |
| Low           | 128                                     | 1.00                           | 3.00                              | 32.0                                        |
| X-Lo          | 32.0                                    | 1.00                           | 3.00                              | 8.00                                        |

## I. GENERATION OF RECOVERY SAMPLES

WR 242511 recovery from plasma extraction was assessed at four different concentrations by comparing the WR 242511 to internal standard peak height ratios in reference samples to the peak height ratios in plasma. Plasma (0.5 ml) samples were spiked with WR 242511 (and vortexed) then prepared as described above in "Sample Preparation," except no standard curve is used and the internal standard was added just prior to the evaporation (step 9). Reference samples were generated by preparing 0.5 ml plasma as described above in "Sample Preparation," except that WR 242511 was spiked and internal standard added to the supernatant just prior to the evaporation.

## J. GENERATION OF STABILITY SAMPLES

Long term, system and bench top stability samples were generated in the same way as precision control samples. Long term stability samples were kept at -70°C or -20°C until prepared and analyzed. System stability samples were prepared as described above in "Sample Preparation," were left standing at room temperature up to 4 days, then were kept at -70°C until analyzed. Bench top stability samples were left standing at room temperature up to 6 hours then were kept at -70°C until prepared and analyzed.

The effect of repeated freeze (at -70°C) and thaw (at room temperature) cycles on stabilities of WR 242511 in human plasma samples was determined as follows: A spiked high concentration pooled biological sample (spiked with 20  $\mu$ l of 128  $\mu$ g/ml WR 242511 working solution, q.s. with human plasma to 10 ml and vortexed 1

min = 256 ng/ml) was diluted with blank human plasma (1.25 ml of the 256 ng/ml sample q.s. to 10 ml with blank human plasma to make 32.0 ng/ml) to make a low concentration pooled biological sample. These samples were subjected to five thaw/freeze cycles. For each cycle, a duplicate set of thaw/freeze samples (0.5 ml) was generated at each concentration. The study is run with the following procedure:

- a. Prepare high and low concentration samples labeled H-1, H-2 ... H-5, and L-1, L-2 ... L-5, in duplicate.
- b. Store all samples until frozen at the specified temperature.
- c. Repeatedly thaw and refreeze samples according to the following table. Thaw as if for sample preparation to room temperature. Let thawed samples stand at room temperature for 1 h.

| Cycle | Keep these<br>samples in freezer | Thaw these<br>samples |
|-------|----------------------------------|-----------------------|
| 1     | 1                                | 2, 3, 4, 5            |
| 2     | 1, 2                             | 3, 4, 5               |
| 3     | 1, 2, 3                          | 4, 5                  |
| 4     | 1, 2, 3, 4                       | 5                     |
| 5     | 1, 2, 3, 4, 5                    | none                  |

- d. Following Cycle 5, take out all of the samples, thaw to room temperature, and assay the samples with a standard curve.

## K. VALIDATION RESULTS

### 1. STANDARD CURVE

Chromatograms for each point in a representative standard curve for WR 242511 appear in Figure 3. Peak height ratios for these calibrators appear in Table 1. Statistical parameters of human plasma interday precision standard curve calibrators appear in Table 2. Statistical parameters of dog plasma interday precision standard curve calibrators appear in Table 11.

### 2. INTRA- AND INTERDAY PRECISION

Results for these evaluations appear in Table 3 for human plasma and Table 12 for dog plasma.

### 3. LLOQ

Results for this evaluation appear in Table 4 for human plasma.

#### 4. RECOVERY

Results for this evaluation appear in Table 5 for human plasma and Table 13 for dog plasma.

#### 5. STABILITY

- a. System Stability: Results appear in Table 6.
- b. Long Term Stability: Results appear in Table 7.
- c. Bench Top Stability: Results appear in Table 8.
- d. Freeze/Thaw Stability: Results appear in Table 9.

#### 6. BLIND SAMPLE ANALYSIS

Results appear in Table 10.

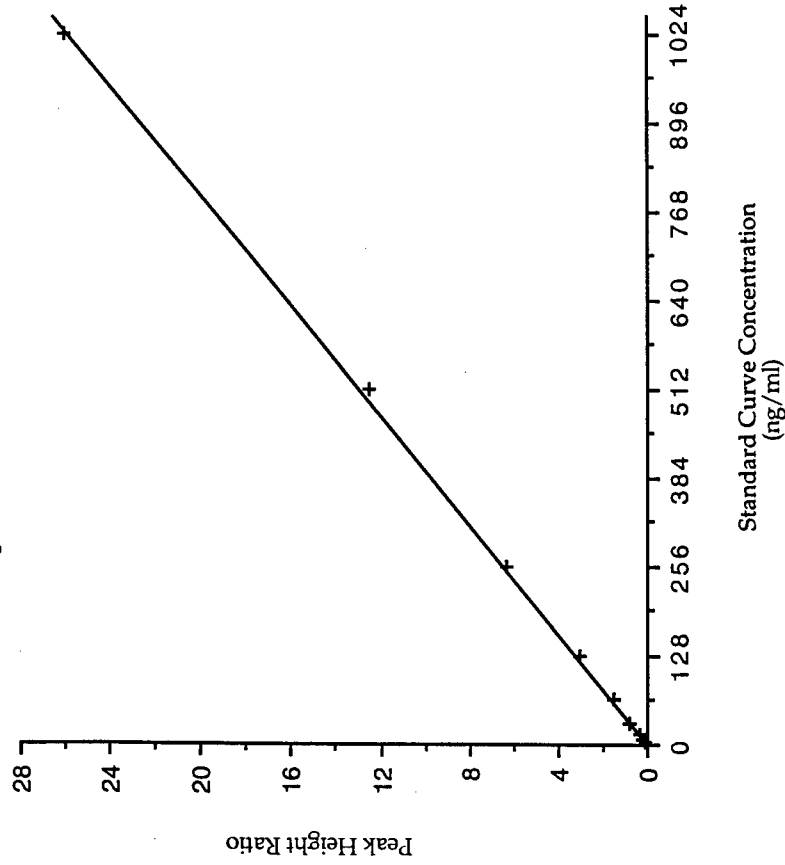
TABLE 1: REPRESENTATIVE STANDARD CURVE FOR  
WR 242,511 PLASMA ASSAY, STUDY REPORT 26

| SPIKED OR<br>DILUTION<br>AMOUNT<br>(ng)* | STANDARD<br>CURVE<br>CONCENTRATION<br>(ng/ml) | PEAK<br>HEIGHT<br>RATIO** | CALCULATED<br>CONCENTRATION<br>(ng/ml) |
|------------------------------------------|-----------------------------------------------|---------------------------|----------------------------------------|
| 0                                        | 0                                             | -                         | -                                      |
| 2.00                                     | 4.00                                          | 0.081                     | 4.21                                   |
| 4.00                                     | 8.00                                          | 0.184                     | 8.32                                   |
| 8.00                                     | 16.0                                          | 0.361                     | 15.4                                   |
| 16.0                                     | 32.0                                          | 0.772                     | 31.8                                   |
| 32.0                                     | 64.0                                          | 1.575                     | 63.9                                   |
| 64.0                                     | 128                                           | 3.023                     | 122                                    |
| 128                                      | 256                                           | 6.373                     | 255                                    |
| 256                                      | 512                                           | 12.543                    | 502                                    |
| 512                                      | 1024                                          | 26.073                    | 1040                                   |

Regression equation:\*\*\*

$$y = 0.0250x - 0.0243, r^2 = 0.9995$$

Representative Standard Curve



\* In 0.5 ml of biological sample. Highest concentration sample obtained by spiking plasma with 40  $\mu$ l of 128  $\mu$ g/ml WR 242511 tartrate working solution (free base concentration), q.s. with plasma to 5 ml and vortexing 1 min. Remaining calibrators obtained by serial dilution.

\*\* Ratio of drug peak height to internal standard peak height.

\*\*\* Standard curve calculated by weighted linear regression where weight =  $1/y_i$ .

**TABLE 2: PRECISION STANDARD CURVE DATA FOR WR 242511 HUMAN PLASMA ASSAY, STUDY REPORT 26**

WR 242511 Standard Curve Parameters

| Validation Run Date | Validation Run No. | Slope      | Intercept  | Coefficient of Determination |
|---------------------|--------------------|------------|------------|------------------------------|
| 10/7/95             | 6                  | 0.02503981 | -0.0243448 | 0.99952132                   |
| 10/10/95            | 7                  | 0.02212795 | -0.0223938 | 0.99803856                   |
| 10/11/95            | 8                  | 0.02221112 | -0.0277856 | 0.99812931                   |
| 10/11/95            | 9                  | 0.02385493 | -0.0323234 | 0.99793302                   |
| 10/12/95            | 10                 | 0.02431651 | -0.0284166 | 0.99595722                   |
| 10/12/95            | 11                 | 0.02419542 | -0.0072980 | 0.99841254                   |
| 12/15/95            | 16                 | 0.02559090 | -0.0465234 | 0.99922106                   |

WR 242511 Back Calculated Standard Calibrators

| Run Number                            | Spiked Concentration (ng/mL) |       |       |       |       |       |        |       |      |
|---------------------------------------|------------------------------|-------|-------|-------|-------|-------|--------|-------|------|
|                                       | 4.00                         | 8.00  | 16.0  | 32.0  | 64.0  | 128   | 256    | 512   | 1024 |
| Back Calculated Concentration (ng/mL) |                              |       |       |       |       |       |        |       |      |
| 6                                     | 4.21                         | 8.32  | 15.4  | 31.8  | 63.9  | 122   | 255    | 502   | 1040 |
| 7                                     | 4.72                         | 8.33  | 15.3  | 29.9  | 58.9  | 116   | 277    | 509   | 1030 |
| 8                                     | 4.81                         | 8.90  | 15.0  | 28.1  | 57.2  | 132   | 241    | 511   | 1050 |
| 9                                     | 4.83                         | 8.19  | 14.9  | 30.1  | 58.2  | 119   | 259    | 545   | 1010 |
| 10                                    | 4.29                         | 9.31  | 16.6  | 30.3  | 62.4  | 103   | 249    | 532   | 1040 |
| 11                                    | 4.56                         | 7.78  | 15.4  | 33.7  | 58.8  | 132   | 245    | 493   | 1060 |
| 16                                    | 4.87                         | 7.87  | 15.2  | 29.8  | 58.6  | 124   | 253    | 509   | 1040 |
| n                                     | 7                            | 7     | 7     | 7     | 7     | 7     | 7      | 7     | 7    |
| Mean                                  | 4.61                         | 8.39  | 15.4  | 30.5  | 59.7  | 121   | 254    | 514   | 1040 |
| SD                                    | 0.269                        | 0.547 | 0.563 | 1.77  | 2.45  | 10.0  | 11.8   | 17.9  | 15.7 |
| Percent CV                            | 5.82                         | 6.53  | 3.65  | 5.79  | 4.11  | 8.29  | 4.63   | 3.48  | 1.52 |
| Percent RE                            | 15.3                         | 4.82  | -3.75 | -4.60 | -6.70 | -5.36 | -0.725 | 0.474 | 1.42 |

TABLE 3: PRECISION OF WR 242511 HUMAN PLASMA ASSAY

Interday Precision WR 242511

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |       |       |        |
|---------------------------------|------------------|-------------------------------|-------|-------|--------|
|                                 |                  | 8.00                          | 32.0  | 128   | 256    |
| Measured Concentrations (ng/mL) |                  |                               |       |       |        |
| 6                               | 1                | 7.84                          | 26.2  | 106   | 224    |
|                                 | 2                | 7.76                          | 27.6  | 113   | 216    |
| 7                               | 1                | 8.83                          | 30.4  | 117   | 240    |
|                                 | 2                | 8.78                          | 27.7  | 107   | 260    |
| 8                               | 1                | 8.81                          | 28.0  | 121   | 245    |
|                                 | 2                | 7.60                          | 30.6  | 117   | 229    |
| 9                               | 1                | 9.45                          | 35.4  | 133   | 277    |
|                                 | 2                | 7.89                          | 37.0  | 153   | 270    |
| 10                              | 1                | 9.35                          | 35.5  | 133   | 274    |
|                                 | 2                | 9.39                          | 35.6  | 132   | 287    |
| 11                              | 1                | 7.66                          | 29.7  | 128   | 268    |
|                                 | 2                | 7.82                          | 31.5  | 125   | 273    |
| n                               |                  | 12                            | 12    | 12    | 12     |
| Mean                            |                  | 8.43                          | 31.3  | 124   | 255    |
| SD                              |                  | 0.737                         | 3.73  | 13.2  | 23.5   |
| Percent CV                      |                  | 8.74                          | 11.9  | 10.7  | 9.22   |
| Percent RE                      |                  | 5.40                          | -2.29 | -3.32 | -0.293 |

Intraday Precision WR 242511

| Validation<br>Run No.           | QC<br>Sample No. | Spiked Concentrations (ng/mL) |      |      |      |
|---------------------------------|------------------|-------------------------------|------|------|------|
|                                 |                  | 8.00                          | 32.0 | 128  | 256  |
| Measured Concentrations (ng/mL) |                  |                               |      |      |      |
| 16                              | 1                | 8.66                          | 36.9 | 146  | 280  |
|                                 | 2                | 9.09                          | 33.2 | 138  | 283  |
|                                 | 3                | 8.81                          | 31.4 | bc   | 260  |
|                                 | 4                | 8.46                          | 32.8 | 136  | 272  |
|                                 | 5                | 9.16                          | 35.1 | 136  | 270  |
|                                 | 6                | 9.71                          | 32.6 | 138  | 261  |
| n                               |                  | 6                             | 6    | 5    | 6    |
| Mean                            |                  | 8.98                          | 33.7 | 139  | 271  |
| SD                              |                  | 0.443                         | 1.99 | 4.15 | 9.47 |
| Percent CV                      |                  | 4.93                          | 5.90 | 2.99 | 3.49 |
| Percent RE                      |                  | 12.3                          | 5.21 | 8.44 | 5.86 |

bc = Unacceptable chromatogram.

**TABLE 4: LOWER LIMIT OF QUANTITATION OF THE HUMAN PLASMA  
ASSAY FOR WR 242511**

| Spiked Concentration |  | 4.00 ng/ml             | 4.00 ng/ml |
|----------------------|--|------------------------|------------|
|                      |  | Back Calculated        | Measured   |
| Sample               |  | Concentrations (ng/ml) |            |
|                      |  | Interday               | Intraday   |
| 1                    |  | 4.21                   | 3.69       |
| 2                    |  | 4.72                   | 3.06       |
| 3                    |  | 4.81                   | 3.33       |
| 4                    |  | 4.83                   | 3.69       |
| 5                    |  | 4.29                   | 3.15       |
| 6                    |  | 4.56                   | 3.33       |
| Mean                 |  | 4.57                   | 3.38       |
| SD                   |  | 0.267                  | 0.265      |
| Percent CV           |  | 5.84                   | 7.87       |
| Percent RE           |  | 14.2                   | -15.6      |

TABLE 5: RECOVERY OF WR 242511 FROM HUMAN PLASMA

| SAMPLE ID   | SPIKED CONCENTRATION Range | PEAK HEIGHT RATIO |              | MEAN PERCENT RECOVERY |
|-------------|----------------------------|-------------------|--------------|-----------------------|
|             |                            | SOLVENT           | PLASMA       |                       |
| 1           | X Low                      | 0.198             | 0.181        | 75.6                  |
| 2           |                            | 0.211             | 0.149        |                       |
| 3           |                            | bc                | 0.134        |                       |
| Mean (± SD) |                            | 0.205 ±0.009      | 0.155 ±0.024 |                       |
| 1           | Low                        | 0.875             | 0.629        | 74.1                  |
| 2           |                            | 0.829             | 0.6454       |                       |
| 3           |                            | 0.869             | 0.633        |                       |
| Mean (± SD) |                            | 0.858 ±0.025      | 0.636 ±0.009 |                       |
| 1           | Medium                     | 3.458             | 2.629        | 76.4                  |
| 2           |                            | 3.434             | 2.622        |                       |
| 3           |                            | 3.14              | 2.412        |                       |
| Mean (± SD) |                            | 3.344 ±0.177      | 2.554 ±0.123 |                       |
| 1           | High                       | 6.248             | 5.090        | 82.3                  |
| 2           |                            | 5.908             | 5.208        |                       |
| 3           |                            | 6.735             | 5.242        |                       |
| Mean (± SD) |                            | 6.297 ±0.416      | 5.180 ±0.080 |                       |
| AVERAGE =   |                            |                   |              | 77.1                  |

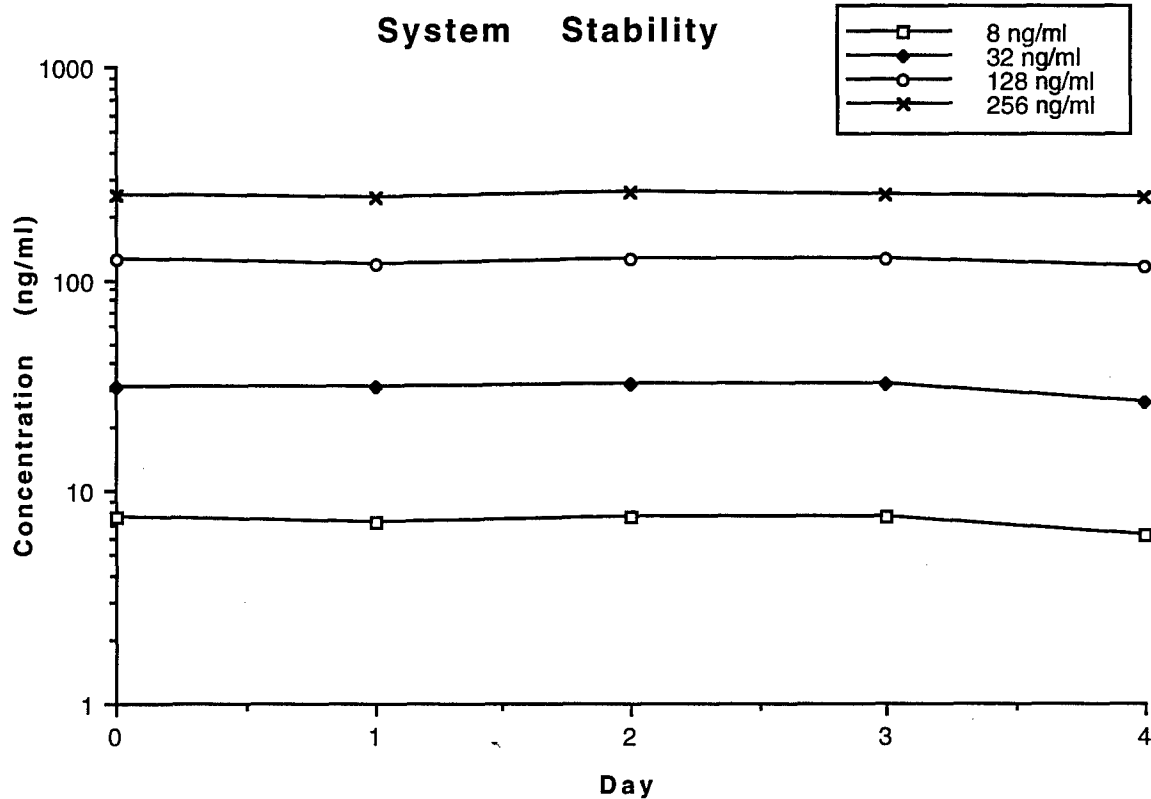
bc = Unacceptable chromatogram.

TABLE 6: SYSTEM STABILITY IN PREPARED HUMAN PLASMA

Concentration for Prepared Biological Samples Stored at Room Temperature

WR 242511

| Spiked Concentration: | CONCENTRATION* |      |     |     |
|-----------------------|----------------|------|-----|-----|
|                       | (ng/ml)        |      |     |     |
| TIME STORED           | 8.00           | 32.0 | 128 | 256 |
| 0 days                | 7.52           | 31.6 | 124 | 250 |
| 1 day                 | 7.07           | 31.0 | 119 | 243 |
| 2 days                | 7.64           | 32.0 | 124 | 258 |
| 3 days                | 7.66           | 32.2 | 126 | 252 |
| 4 days                | 6.25           | 26.1 | 113 | 242 |

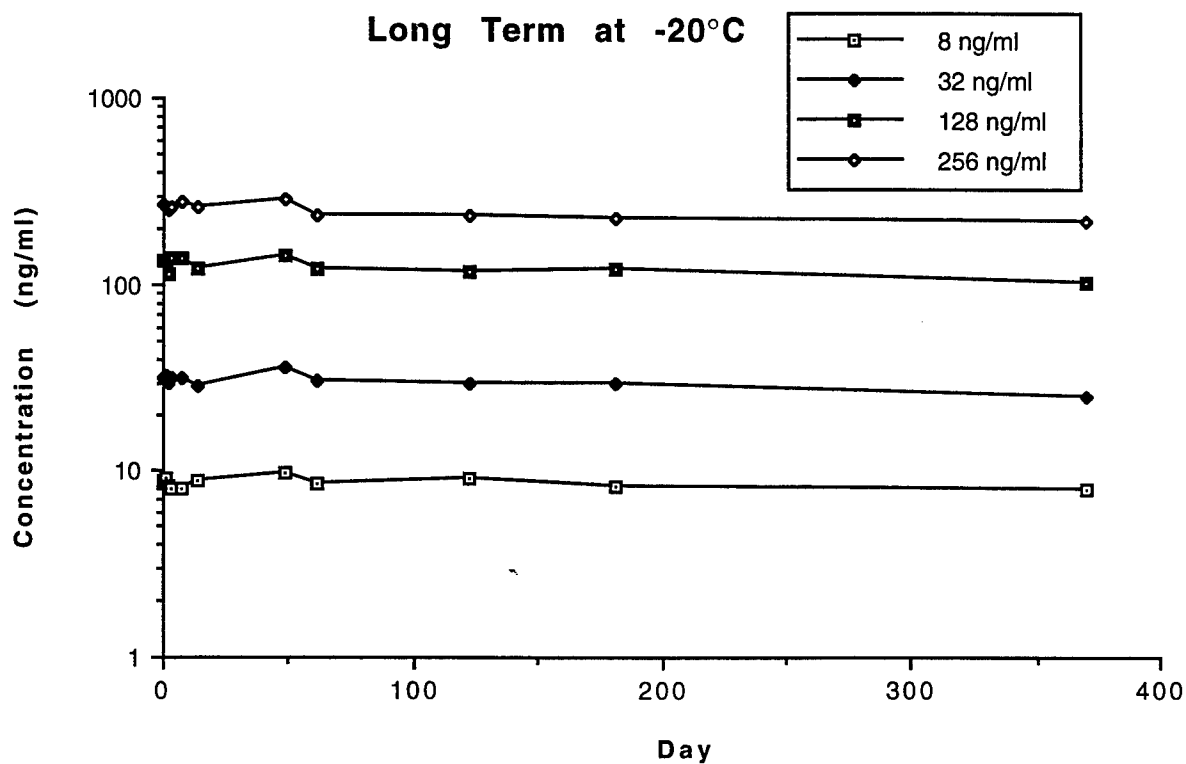


\* Measured concentrations are averages of two analyses.

TABLE 7A: LONG TERM STABILITY OF WR 242511 IN HUMAN PLASMA

WR 242511 CONCENTRATION IN PLASMA STORED AT -20°C

| Spiked Concentration: | CONCENTRATION* (ng/ml) |      |     |     |
|-----------------------|------------------------|------|-----|-----|
|                       | 8.00                   | 32.0 | 128 | 256 |
| TIME STORED           |                        |      |     |     |
| 0 days                | 8.63                   | 32.0 | 136 | 265 |
| 1 day                 | 9.01                   | 32.6 | 136 | 260 |
| 2 days                | 8.14                   | 29.7 | 113 | 250 |
| 3 days                | 8.01                   | 31.2 | 139 | 256 |
| 1 week                | 7.89                   | 31.4 | 138 | 275 |
| 2 weeks               | 8.80                   | 28.8 | 120 | 262 |
| 49 days               | 9.74                   | 36.0 | 145 | 291 |
| 2 months              | 8.58                   | 30.5 | 120 | 236 |
| 4 months              | 8.94                   | 29.7 | 116 | 237 |
| 6 months              | 8.24                   | 29.2 | 121 | 227 |
| 1 year                | 7.92                   | 25.4 | 103 | 221 |

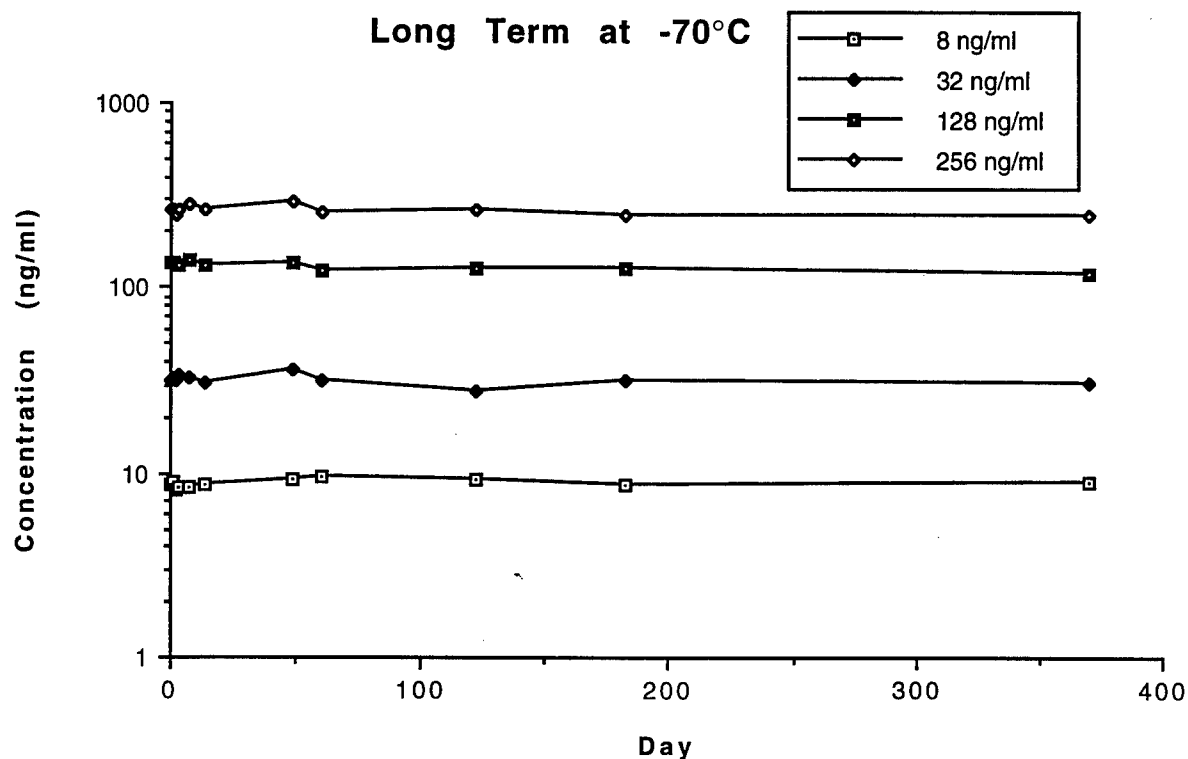


\* Measured concentrations are averages of two analyses.

TABLE 7B: LONG TERM STABILITY OF WR 242511 IN HUMAN PLASMA

WR 242511 CONCENTRATION IN PLASMA STORED AT -70°C

| Spiked Concentration: | CONCENTRATION* (ng/ml) |      |     |     |
|-----------------------|------------------------|------|-----|-----|
|                       | 8.00                   | 32.0 | 128 | 256 |
| TIME STORED           |                        |      |     |     |
| 0 days                | 8.63                   | 32.0 | 136 | 265 |
| 1 day                 | 9.09                   | 32.7 | 135 | 263 |
| 2 days                | 8.16                   | 31.7 | 132 | 249 |
| 3 days                | 8.45                   | 33.5 | 134 | 264 |
| 1 week                | 8.42                   | 32.3 | 139 | 285 |
| 2 weeks               | 8.78                   | 31.0 | 130 | 261 |
| 49 days               | 9.31                   | 36.7 | 135 | 290 |
| 2 month               | 9.64                   | 31.9 | 122 | 253 |
| 4 months              | 9.16                   | 28.0 | 127 | 261 |
| 6 months              | 8.73                   | 31.4 | 127 | 249 |
| 1 year                | 8.82                   | 30.8 | 118 | 244 |

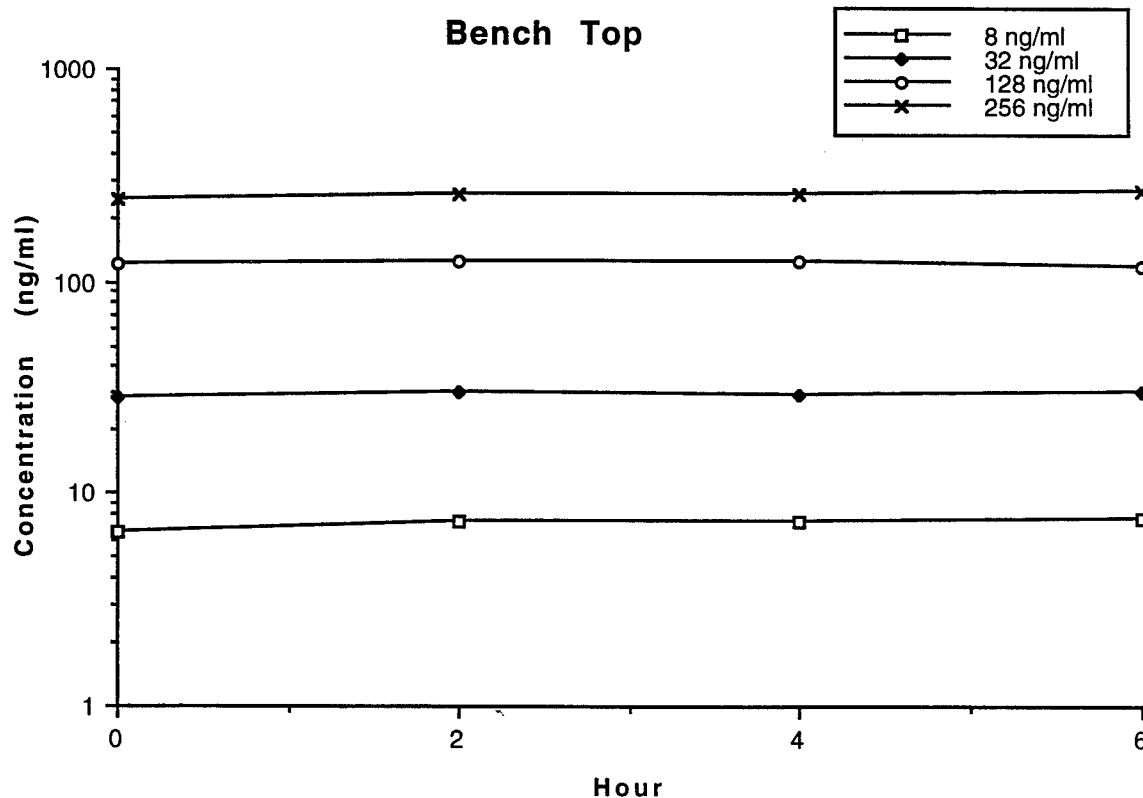


\* Measured concentrations are averages of two analyses.

**TABLE 8: BENCH TOP STABILITY OF WR 242511 IN SPIKED HUMAN PLASMA #**

CONCENTRATION IN PLASMA STORED AT ROOM TEMPERATURE

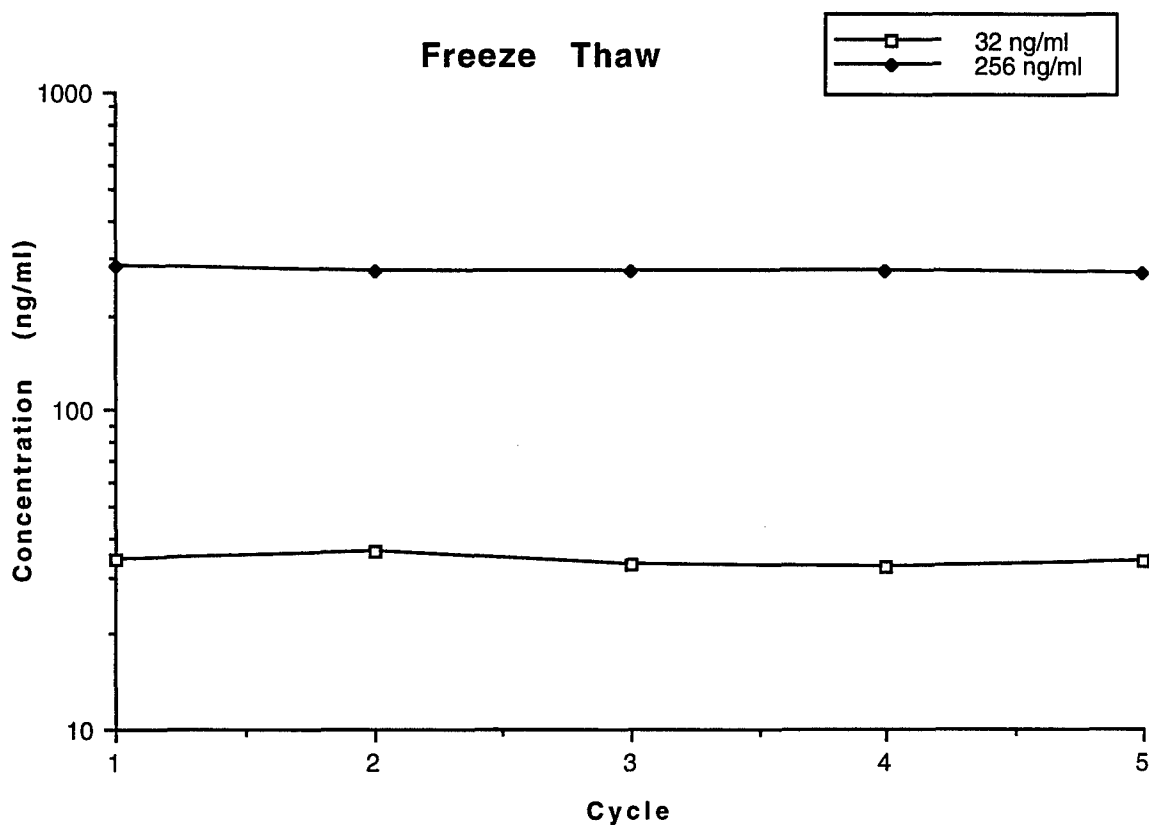
| Spiked Concentration: | CONCENTRATION<br>(ng/ml) |      |     |     |
|-----------------------|--------------------------|------|-----|-----|
|                       | 8.00                     | 32.0 | 128 | 256 |
| TIME STORED           |                          |      |     |     |
| 0 hour                | 6.63                     | 28.7 | 120 | 240 |
| 2 hour                | 7.36                     | 30.6 | 126 | 257 |
| 4 hour                | 7.29                     | 29.4 | 124 | 259 |
| 6 hour                | 7.62                     | 30.0 | 118 | 265 |



# Measured concentrations are averages of two analyses.

**TABLE 9: EFFECT OF REPEATED FREEZE AND THAW CYCLES ON WR 242511 SPIKED HUMAN PLASMA SAMPLES@ #**

| Spiked<br>Concentration | AT ROOM TEMPERATURE                  |                                      |
|-------------------------|--------------------------------------|--------------------------------------|
|                         | Low<br>Concentration<br>(32.0 ng/ml) | High<br>Concentration<br>(256 ng/ml) |
| Cycle                   |                                      |                                      |
| 1                       | 34.4                                 | 284                                  |
| 2                       | 36.4                                 | 274                                  |
| 3                       | 32.8                                 | 277                                  |
| 4                       | 32.2                                 | 275                                  |
| 5                       | 33.5                                 | 271                                  |



@ Individually spiked samples.

# Measured concentrations are averages of two analyses.

**TABLE 10: ACCURACY OF WR 242511 HUMAN PLASMA ASSAY (BLIND STUDY RESULTS)**

| Sample Number | Spiked Level (ng/ml) | Measured Level <sup>#</sup> (ng/ml) | Statistics (ng/ml) |
|---------------|----------------------|-------------------------------------|--------------------|
| 4             | 0                    | 6.48 <sup>@</sup>                   |                    |
| 9             |                      | *                                   |                    |
| 13            |                      | *                                   |                    |
| 23            |                      | *                                   |                    |
| 30            |                      | *                                   |                    |
| 7             | 4.70                 | 5.93                                | Mean = 5.73        |
| 15            |                      | 6.39                                | SD = 0.55          |
| 20            |                      | 5.13                                | Percent CV = 9.61  |
| 24            |                      | 5.46                                | Percent RE = 21.9  |
| 27            |                      | * <sup>@</sup>                      |                    |
| 1             | 25.9                 | 26.7                                | Mean = 27.0        |
| 3             |                      | 26.6                                | SD = 0.635         |
| 11            |                      | 26.6                                | Percent CV = 2.36  |
| 17            |                      | 43.0 <sup>@</sup>                   | Percent RE = 4.05  |
| 29            |                      | 27.9                                |                    |
| 5             | 112                  | 103                                 | Mean = 103         |
| 10            |                      | 103                                 | SD = 1.82          |
| 18            |                      | 102                                 | Percent CV = 1.77  |
| 21            |                      | 105                                 | Percent RE = -8.39 |
| 28            |                      | 100                                 |                    |
| 6             | 415                  | 390                                 | Mean = 389         |
| 8             |                      | 392                                 | SD = 7.81          |
| 12            |                      | 397                                 | Percent CV = 2.01  |
| 19            |                      | 390                                 | Percent RE = -6.27 |
| 26            |                      | 376                                 |                    |
| 2             | 822                  | 793                                 | Mean = 793         |
| 14            |                      | 802                                 | SD = 7.60          |
| 16            |                      | 791                                 | Percent CV = 0.958 |
| 22            |                      | 782                                 | Percent RE = -3.50 |
| 25            |                      | 798                                 |                    |

<sup>#</sup> Measured concentrations are averages of three analyses.

<sup>@</sup> Anomalous result: samples 4 and 27 appear to have been switched or mislabeled and sample 17 has bad precision. Results for these three samples were not included in CV and RE calculations.

\* = Below assay sensitivity.

**TABLE 11: PRECISION STANDARD CURVE DATA FOR WR 242511 DOG  
PLASMA ASSAY, STUDY REPORT 26**

WR 242511 Standard Curve Parameters

| Validation Run<br>Date | Validation Run<br>No. | Slope      | Intercept  | Coefficient of<br>Determination |
|------------------------|-----------------------|------------|------------|---------------------------------|
| 9/4/96                 | inter1                | 0.02111344 | -0.0295347 | 0.99857702                      |
| 9/5/96                 | intra                 | 0.02444799 | -0.0069828 | 0.99891019                      |
| 9/12/96                | inter2                | 0.02755975 | -0.0016602 | 0.99275586                      |
| 9/13/96                | inter3                | 0.02671813 | -0.0111891 | 0.99343441                      |

WR 242511 Back Calculated Standard Calibrators

| Run<br>Number | Spiked Concentration (ng/mL)          |       |       |      |       |       |       |       |      |
|---------------|---------------------------------------|-------|-------|------|-------|-------|-------|-------|------|
|               | 4.00                                  | 8.00  | 16.0  | 32.0 | 64.0  | 128   | 256   | 512   | 1024 |
|               | Back Calculated Concentration (ng/mL) |       |       |      |       |       |       |       |      |
| inter1        | 4.76                                  | 8.50  | 14.4  | 29.6 | 57.8  | 134   | 251   | 498   | 1050 |
| intra         | 4.25                                  | 8.30  | 16.2  | 32.1 | 63.0  | 120   | 245   | 503   | 1050 |
| inter2        | 4.12                                  | 8.22  | 14.9  | 42.3 | 61.8  | 114   | 242   | 471   | 1100 |
| inter3        | 4.54                                  | 7.94  | 14.6  | 41.0 | 61.5  | 112   | 242   | 477   | 1090 |
| n             | 4                                     | 4     | 4     | 4    | 4     | 4     | 4     | 4     | 4    |
| Mean          | 4.42                                  | 8.24  | 15.0  | 36.3 | 61.0  | 120   | 245   | 487   | 1070 |
| SD            | 0.288                                 | 0.232 | 0.81  | 6.34 | 2.25  | 9.93  | 4.24  | 15.6  | 26.3 |
| Percent CV    | 6.52                                  | 2.82  | 5.39  | 17.5 | 3.68  | 8.28  | 1.73  | 3.21  | 2.45 |
| Percent RE    | 10.4                                  | 3.00  | -6.09 | 13.3 | -4.65 | -6.25 | -4.30 | -4.83 | 4.74 |

TABLE 12: PRECISION OF WR 242511 DOG PLASMA ASSAY

Interday Precision WR 242511

| Validation                      | QC         | Spiked Concentrations (ng/mL) |       |       |       |
|---------------------------------|------------|-------------------------------|-------|-------|-------|
| Run No.                         | Sample No. | 8.00                          | 32.0  | 128   | 256   |
| Measured Concentrations (ng/mL) |            |                               |       |       |       |
| inter1                          | 1          | 9.02                          | 30.5  | 115   | 224   |
|                                 | 2          | 8.60                          | 30.6  | 123   | 262   |
| inter2                          | 1          | 8.51                          | 29.2  | 133   | 250   |
|                                 | 2          | 6.23                          | 31.9  | 123   | 234   |
| inter3                          | 1          | 8.43                          | 28.3  | 134   | 252   |
|                                 | 2          | 7.16                          | 30.4  | 122   | 238   |
|                                 | n          | 6                             | 6     | 6     | 6     |
|                                 | Mean       | 7.99                          | 30.2  | 125   | 243   |
|                                 | SD         | 1.07                          | 1.25  | 7.24  | 13.8  |
|                                 | Percent CV | 13.3                          | 4.14  | 5.79  | 5.69  |
|                                 | Percent RE | -0.104                        | -5.78 | -2.34 | -4.95 |

Intraday Precision WR 242511

| Validation                      | QC         | Spiked Concentrations (ng/mL) |       |       |       |
|---------------------------------|------------|-------------------------------|-------|-------|-------|
| Run No.                         | Sample No. | 8.00                          | 32.0  | 128   | 256   |
| Measured Concentrations (ng/mL) |            |                               |       |       |       |
| intra                           | 1          | 7.20                          | 27.9  | 114   | 231   |
|                                 | 2          | 8.14                          | 29.0  | 115   | 231   |
|                                 | 3          | 7.61                          | 29.1  | 113   | 233   |
|                                 | 4          | 8.26                          | 28.9  | 116   | 229   |
|                                 | 5          | 7.57                          | 28.8  | 114   | 228   |
|                                 | 6          | 7.61                          | 28.5  | 113   | 231   |
|                                 | n          | 6                             | 6     | 6     | 6     |
|                                 | Mean       | 7.73                          | 28.7  | 114   | 231   |
|                                 | SD         | 0.396                         | 0.443 | 1.17  | 1.76  |
|                                 | Percent CV | 5.12                          | 1.54  | 1.02  | 0.764 |
|                                 | Percent RE | -3.35                         | -10.3 | -10.8 | -9.96 |

TABLE 13: RECOVERY OF WR 242511 FROM DOG PLASMA

| SAMPLE ID   | SPIKED CONCENTRATION Range | PEAK HEIGHT RATIO |              | MEAN PERCENT RECOVERY |
|-------------|----------------------------|-------------------|--------------|-----------------------|
|             |                            | SOLVENT           | PLASMA       |                       |
| 1           | X Low                      | 0.140             | 0.114        | 73.9                  |
| 2           |                            | 0.206             | 0.134        |                       |
| 3           |                            | 0.129             | 0.103        |                       |
| Mean (± SD) |                            | 0.158 ±0.042      | 0.117 ±0.016 |                       |
| 1           | Low                        | 0.541             | 0.510        | 87.4                  |
| 2           |                            | 0.521             | 0.495        |                       |
| 3           |                            | 0.563             | 0.415        |                       |
| Mean (± SD) |                            | 0.542 ±0.021      | 0.473 ±0.051 |                       |
| 1           | Medium                     | 2.279             | 2.008        | 75.2                  |
| 2           |                            | 3.069             | 1.871        |                       |
| 3           |                            | 2.484             | 2.011        |                       |
| Mean (± SD) |                            | 2.611 ±0.410      | 1.963 ±0.080 |                       |
| 1           | High                       | 5.261             | 4.022        | 80.8                  |
| 2           |                            | 5.943             | 4.578        |                       |
| 3           |                            | 4.727             | 4.278        |                       |
| Mean (± SD) |                            | 5.310 ±0.609      | 4.293 ±0.278 |                       |
| AVERAGE =   |                            |                   |              | 79.3                  |