ATS LABS

FINAL STUDY REPORT

PROTOCOL TITLE

Evaluation of Antiviral Properties of a Product Using a Virucidal Suspension Assay

Virus: Human Coronavirus

PRODUCT IDENTITY

Microcyn 60
Lot # S120104-03

PROTOCOL NUMBER

SRC26120104.HCV

PROJECT NUMBER

A02569

AUTHOR

Karen M. Ramm, B.A.
Study Director

STUDY COMPLETION DATE

January 10, 2005

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

SPONSOR

Oculus Innovative Sciences, Inc.
1129 N. McDowell Blvd.
Petaluma, CA 94954

SPONSOR REPRESENTATIVE

Scientific & Regulatory Consultants, Inc.
102 1/2 South Chauncey Street
Columbia City, IN 46725-2306

GOOD LABORATORY PRACTICE STATEMENT

The study referenced in this report was conducted in compliance with U.S. Environmental Protection Agency and U.S. Food and Drug Administration Good Laboratory Practice (GLP) regulations set forth in 40 CFR part 160 and 21 CFR part 58 respectively.

The studies not performed by or under the direction of ATS Labs are exempt from this Good Laboratory Practice statement and include: characterization and stability of the compound(s).

Submitter: _____ _____
Date

Sponsor: Rhonda Jones _____ 1-13-05
Rhonda Jones, Agent for Oculus Innovative Sciences, Inc. Date

Study Director: Karen M. Ramm _____ 1-10-05
Karen M. Ramm, B.A. Date

QUALITY ASSURANCE UNIT SUMMARY

Study: Evaluation of Antiviral Properties of a Product Using a Virucidal Suspension Assay

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of nonclinical laboratory studies. These studies have been performed under Good Laboratory Practice regulations (40 CFR Part 160 and 21 CFR Part 58) and in accordance to standard operating procedures and standard protocols. The Quality Assurance Unit maintains copies of study protocols and standard operating procedures and has inspected this study on the date(s) listed below. Studies are inspected at time intervals to assure the integrity of the study.

Phase Inspected	Date	Study Director	Management
Critical Phase	December 16, 2004	December 16, 2004	December 21, 2004
Draft Report	December 21, 2004	December 21, 2004	
Final Report	January 7, 2005	January 7, 2005	January 10, 2005

The findings of these inspections have been reported to management and the Study Director.

Quality Assurance Auditor: Rachelle L Ewman Date: 01/10/05

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STUDY PERSONNEL

STUDY DIRECTOR: Karen M. Ramm, B.A.

Professional personnel involved:

Sandi True, Ph.D.	- Research Scientist I
Katherine A. Paulson, M.L.T.	- Research Assistant II
Adam W. Pitt, B.S.	- Research Assistant II

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Evaluation of Antiviral Properties of a Product Using a Virucidal Suspension Assay

Project Number: A02569

Protocol Number: SRC26120104.HCV

Sponsor: Oculus Innovative Sciences, Inc.
1129 N. McDowell Blvd.
Petaluma, CA 94954

Sponsor Representative: Scientific & Regulatory Consultants, Inc.
102 1/2 South Chauncey Street
Columbia City, IN 46725-2306

Testing Facility: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

TEST SUBSTANCE IDENTITY

Test Substance: Microcyn 60

Lot/Batch(s): Lot # S120104-03

Test Substance Characterization
Test substance characterization as to content, stability, solubility, storage, etc., is the responsibility of the Sponsor. (Sponsor Test Material Characterization Report, Attachment I)

STUDY DATES

Date Sample Received: December 3, 2004
Study Initiation Date: December 3, 2004
Experimental Start Date: December 6, 2004
Experimental End Date: December 16, 2004
Study Completion Date: January 10, 2005

OBJECTIVE

The purpose of this study was to evaluate the antiviral properties of a product against Human Coronavirus when exposed (in suspension) for the specified exposure periods. The protocol is a modification of the Standard Test Method for Efficacy of Virucidal Agents Intended for Special Applications (ASTM E1052).

SUMMARY OF RESULTS

Test Substance:	Microcyn 60 , Lot # S120104-03
Dilution Tested:	Ready to use (RTU)
Virus:	Human Coronavirus, Strain 229E, ATCC VR-740
Exposure Time(s):	15 seconds, 30 seconds and 60 seconds
Exposure Temperature:	25±1°C
Organic Soil Load:	1% fetal bovine serum
Efficacy Result:	Under these test conditions, Microcyn 60 (Lot # S120104-03) demonstrated a ≥99.9% reduction in the stock virus titer following 15 second and 30 second exposure times. The log reduction was ≥3.0 $\log_{10}$. Under these test conditions, Microcyn 60 (Lot #S120104-03) demonstrated a ≥99.94% reduction in the stock virus titer following a 60 second exposure time. The log reduction was ≥3.25 $\log_{10}$.

TEST SYSTEM

1. Virus
   The 229E strain of Human Coronavirus used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-740). Stock virus was prepared by collecting the supernatant culture fluid from infected culture cells. The cells were disrupted and cell debris removed by centrifugation at approximately 1800 RPM for approximately five minutes at approximately 4°C. The supernatant was removed, aliquoted, and the high titer stock virus was stored at ≤ -70°C until the day of use. On the day of use an aliquot of stock virus (ATS Labs Lot HCV-30) was removed, thawed and refrigerated until use in the assay. The stock virus culture was adjusted to contain 1% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Human Coronavirus on MRC-5 cells.

2. Test Cell Cultures
   Cultures of MRC-5 (human embryonic lung) cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL-171). The cells were propagated by ATS Labs personnel. The cells were seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2.

3. Test Medium
   Test medium used in this study was Minimum essential medium (MEM) supplemented with 2% heat-inactivated fetal bovine serum (FBS), 10 µg/mL gentamicin, 100 units/mL penicillin, and 2.5 µg/mL amphotericin B.

The following table lists the test and control groups, the dilutions assayed, and the number of cultures used. See the report text for a more detailed explanation.

NUMBER OF DILUTIONS AND CULTURES FOR VIRUCIDAL SUSPENSION STUDY			
Test or Control Group	**Dilutions Assayed (log_{10})**	**Cultures per dilution**	**Total Cultures**
Cell Control	N/A	4	4/group
Virus Control (for each exposure time)	-2,-3,-4,-5,-6	4	20
Sample batch + virus (for each exposure time)	-2,-3,-4,-5,-6	4	20
Cytotoxicity Control	-2,-3,-4,-5,-6	4	20
Neutralization Control	-2,-3,-4,-5,-6	4	20

METHODS

1. Preparation of Test Substance
   Microcyn 60 was used as it was received from the Sponsor. The test substance removed from the original container was in solution as determined by visual observation.

2. Treatment of Virus Suspension
   A 4.5 mL aliquot of the test substance was dispensed into a sterile 15 mL conical tube and mixed with a 0.5 mL aliquot of the stock virus suspension. The mixture was vortex mixed for a minimum of 10 seconds and held for the remainder of the specified exposure times at room temperature (24.5°C). The exposure times assayed were 15 seconds, 30 seconds, and 60 seconds. Immediately following each exposure period, a 0.1 mL aliquot was removed and the mixtures were titered by 10-fold serial dilutions (0.1 mL + 0.9 mL test medium) and assayed for the presence of virus. Note: To decrease the test substance cytotoxicity, the first dilution was made in FBS with the remaining dilutions in test medium.

3. Treatment of Virus Control
   A 0.5 mL aliquot of stock virus suspension was exposed to a 4.5 mL aliquot of test medium instead of test substance and treated as previously described. (All controls employed the FBS neutralizer as described in the Treatment of Virus Suspension section). A virus control was performed for each exposure time. The virus control titer was used as a baseline to compare the percent and log reduction of each test parameter following exposure to the test substance.

4. Cytotoxicity Controls
   A 4.5 mL aliquot of the test substance was mixed with a 0.5 mL aliquot of test medium in lieu of virus and treated as previously described. The cytotoxicity of the cell cultures was scored at the same time as virus-test substance and virus control cultures. Cytotoxicity was graded on the basis of cell viability as determined microscopically. Cellular alterations due to toxicity were graded and reported as toxic (T) if greater than or equal to 50% of the monolayer was affected.

5. Neutralization Control
Each cytotoxicity control mixture (above) was challenged with low titer stock virus to determine the dilution(s) of test substance at which virucidal activity, if any, was retained. Dilutions that showed virucidal activity were not considered in determining reduction of the virus by the test substance.

6. Neutralization
As previously described, 0.1 mL of each test and control parameter following the exposure period was added to fetal bovine serum (0.9 mL) followed immediately by 10-fold serial dilutions in test medium to stop the action of the test substance. To determine if the neutralizer chosen for the assay was effective in diminishing the virucidal activity of the test substance, low titer stock virus was added to each dilution of the test substance-neutralizer mixture. This mixture was assayed for the presence of virus (neutralization control above).

7. Infectivity Assay
The MRC-5 cell line, which exhibits CPE in the presence of Human Coronavirus, was used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes were inoculated in quadruplicate with 0.1 mL of the dilutions prepared from test and control groups. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. The cultures were incubated at 31-35°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures were scored periodically for ten days for the absence or presence of CPE, cytotoxicity, and for viability.

8. Statistical Methods: Not applicable

PROTOCOL CHANGES

Protocol Amendments:
No protocol amendments were required for this study.

Protocol Deviations:
No protocol deviations occurred during this study.

DATA ANALYSIS

Calculations

Viral and cytotoxicity titers are expressed as -$\log_{10}$ of the 50 percent titration endpoint for infectivity ($TCID_{50}$) or cytotoxicity (TCD_{50}), respectively, as calculated by the method of Spearman Karber.

$$\text{- Log of 1st dilution inoculated} - \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100}\right) - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Percent (%) Reduction Formula

$$\% \text{ Reduction} = 1 - \left[\frac{TCID_{50} \text{ test}}{TCID_{50} \text{ virus control}}\right] \times 100$$

Log Reduction Formula

$$\text{Log Reduction} = TCID_{50} \text{ of the virus control} - TCID_{50} \text{ of the test}$$

STUDY RETENTION

Record Retention
All of the original raw data developed exclusively for this study shall be archived at ATS Labs, 1285 Corporate Center Drive, Suite 110, Eagan, MN 55121. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of the final study report.
7. Study-specific SOP deviations made during the study.

Test Substance Retention
The test substance will be discarded following study completion per Sponsor approved protocol. It is the responsibility of the Sponsor to retain a sample of the test material.

REFERENCES

1. Annual Book of ASTM Standards 2000, Section 11 Water and Environmental Technology, Volume 11.05 Biological Effects and Environmental Fate: Biotechnology: Pesticides, E1052-96.

2. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Schmidt, N.J. and Emmons, R.W. editors. Sixth edition, 1989. p. 18-20.

STUDY RESULTS

Cytotoxicity and Neutralization Controls
Test substance cytotoxicity was observed at ≤1.5 log_{10}. The neutralization control demonstrated that the test substance was neutralized at ≤1.5 log_{10}.

15 Second Exposure Period
The titer of the virus control was 4.5 log_{10}. Following exposure, test virus infectivity was not detected in the virus-test substance mixture at any dilution tested (≤1.5 log_{10}).

30 Second Exposure Period
The titer of the virus control was 4.5 log_{10}. Following exposure, test virus infectivity was not detected in the virus-test substance mixture at any dilution tested (≤1.5 log_{10}).

60 Second Exposure Period
The titer of the virus control was 4.75 log_{10}. Following exposure, test virus infectivity was not detected in the virus-test substance mixture at any dilution tested (≤1.5 log_{10}).

STUDY CONCLUSION

Under the conditions of this investigation, in the presence of a 1% fetal bovine serum organic soil load, Microcyn 60 (Lot # S120104-03), ready to use, demonstrated a ≥99.9% reduction in viral titer following a 15 second exposure period (≥3.0 log_{10} reduction), a ≥99.9% reduction in viral titer following a 30 second exposure period (≥3.0 log_{10} reduction) and a ≥99.94% reduction in viral titer following a 60 second exposure period (≥3.25 log_{10} reduction) to Human Coronavirus.

In the opinion of the Study Director, there were no circumstances that may have adversely affected the quality or integrity of the data.

TABLE 1: Results

Effects of Microcyn 60 (Lot # S120104-03) Against Human Coronavirus in Suspension Following 15 Second, 30 Second, and 60 Second Exposure Periods

Dilution	Virus Control			Test: Human Coronavirus + Microcyn 60			Cytotoxicity Control	Neutralization Control
	Exposure Time 15 seconds	Exposure Time 30 seconds	Exposure Time 60 seconds	Exposure Time 15 seconds	Exposure Time 30 seconds	Exposure Time 60 seconds	Microcyn 60	Human Coronavirus + Microcyn 60
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-2}	+ + + +	+ + + +	+ + + +	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	+ + + +
10^{-3}	+ + + +	+ + + +	+ + + +	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	+ + + +
10^{-4}	+ + + +	+ + + +	+ + + +	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	+ + + +
10^{-5}	0 0 0 0	0 0 0 0	0 + 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	+ + + +
10^{-6}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	+ + + +
$TCID_{50}$/0.1 mL	$10^{4.5}$	$10^{4.5}$	$10^{4.75}$	$\leq 10^{1.5}$	$\leq 10^{1.5}$	$\leq 10^{1.5}$	$*\leq 10^{1.5}$	Neutralized at ≤ 1.5 Log_{10}
Percent Reduction	NA	NA	NA	≥99.9%	≥99.9%	≥99.94%	NA	NA
Log_{10} Reduction	NA	NA	NA	≥3.0 log_{10}	≥3.0 log_{10}	≥3.25 log_{10}	NA	NA

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(NA) = Not applicable
(*) = Cytotoxicity results are reported as TCD_{50}/0.1 mL

ATTACHMENT I

Test Material Characterization Report

TEST OBJECTIVE	To determine the pH, ORP and Free Available Chlorine levels of the product sample
TEST MATERIAL	Microcyn™ Lot #S120104-03
SPONSOR	Oculus Innovative Sciences, Inc. 1129 N. McDowell Blvd. Petaluma, CA 94954
TEST FACILITY	Oculus Innovative Sciences, Inc. 1129 N. McDowell Blvd. Petaluma, CA 94954
TEST METHOD	pH was tested using a Thermo-Orion 525+ meter with a Ross pH electrode. The system is calibrated before every reading using buffer solutions and a control check is performed prior to pH measurements being made ORP is measure using a Thermo-Orion 525+ meter with a VWR Symphony electrode. A control is measured prior to each ORP measurement Available chlorine was analyzed using a LaMotte Smart 2 colorimeter as described in EPA test method 330.5. In this method, available chlorine in the test material reacts with DPD (N,N-diethyl-p-phenylenediamine) reagent to form a red color that is proportional to free available chlorine.
TEST DATE	12/02/04
TEST RESULTS	pH = 7.7, ORP = 846 mV, FAC = 65.6 ppm
EXPIRATION DATE	12/02/05
TESTED BY	K. Morel KEITH MOREL DIRECTOR, R&D pp Scott Stern Manufacturing Engineer
DATED	12/02/04

EXACT COPY
INITIALS ___ DATE 1/10/05