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FINAL STUDY REPORT

PROTOCOL TITLE

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Virus: Influenza A virus

DATA REQUIREMENT

U.S. EPA 40 CFR Part 158,
"Data Requirements for Registration"
Pesticide Assessment Guidelines - Subdivision G, 91-2(f)

PRODUCT IDENTITY

AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1),
A 2400 PPM CONCENTRATE
LOT# 2001.042.001 and LOT# 2001.005.001

PROTOCOL NUMBER

IMS01121301.FLU

PROJECT NUMBER

12465

AUTHOR

Mary J. Miller, M.T.
Study Director
Chemical Germicide Testing Services

STUDY COMPLETION DATE

January 17, 2002

PERFORMING LABORATORY

AppTec Laboratory Services
2540 Executive Drive
St. Paul, MN 55120

SPONSOR

Innovative Medical Services
1725 Gillespie Way
El Cajon, CA 92[illegible]

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d) (1) (A), (B), or (C).

Company: Innovative Medical Services

Company Agent: DOVANA BLOUNT

ASSISTANT TO THE PRESIDENT
Title

Signature

Date: 02.09.02

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CERTIFICATION OF GOOD LABORATORY PRACTICE

The study referenced in this report was conducted in compliance with U.S. Environmental Protection Agency Good Laboratory Practice (GLP) regulations set forth in 40 CFR Part 160.

The procedures not performed by or under the direction of AppTec Laboratory Services are exempt from this Good Laboratory Practice Statement and include: characterization and stability of the compound(s).

Study Director: Mary J. Miller, M.T. — Date: 1-17-02

Submitter: [signature] — Date: 02.08.02

Sponsor Representative: [signature] — Date: 02.08.02

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QUALITY ASSURANCE UNIT SUMMARY

Study: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of nonclinical laboratory studies. This study has been performed under Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures and a standard protocol. 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	January 3, 2002	January 3, 2002	January 17, 2002
Final Report	January 15, 2002	January 15, 2002	

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

Quality Assurance Auditor: Johnson Date: 1-17-02

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STUDY PERSONNEL

STUDY DIRECTOR: Mary J. Miller, M.T.

Professional Personnel Involved:

William D. Smith, B.S.	- President
Karen M. Ramm, B.A.	- Division Director
Mary J. Miller, M.T.	- Research Scientist II
Katherine A. Paulson, M.L.T.	- Research Assistant II
Carrey A. Bauer, B.S.	- Research Assistant I

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STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Project Number: 12465

Protocol Number: IMS01121301.FLU

Sponsor: Innovative Medical Services
1725 Gillespie Way
El Cajon, CA 92020

Testing Facility: AppTec Laboratory Services
2540 Executive Drive
St. Paul, MN 55120

TEST SUBSTANCE IDENTITY

Test Substance Name: AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE

Lots: LOT# 2001.042.001 and LOT# 2001.005.001

Test Substance Characterization
Test substance characterization as to content, stability, solubility, storage, etc., is the responsibility of the Sponsor.

STUDY DATES

Date Sample Received: November 20, 2001
Study Initiation Date: December 19, 2001
Experimental Start Date: January 3, 2002
Experimental End Date: January 10, 2002
Study Completion Date: January 17, 2002

OBJECTIVE

The objective of this study was to evaluate the virucidal efficacy of a disinfectant against Influenza A virus according to test criteria and methods approved by the United States Environmental Protection Agency (U.S. EPA) for registration of a product as a virucide.

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SUMMARY OF RESULTS

Test Substance: AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE, LOT# 2001.042.001 and LOT# 2001.005.001

Dilution: No dilution required, used as received from The Sponsor

Virus: Influenza A virus, ATCC VR-544, Strain Hong Kong

Exposure Time: 10 minutes

Exposure Temperature: Room temperature

Organic Soil Load: 1% Fetal bovine serum

Efficacy Result: Two lots of AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE met the test criteria specified in the study protocol. The results indicate **complete inactivation** of Influenza A virus under these test conditions as required by the U.S. EPA for claims of virucidal activity.

TEST SYSTEM

1. Virus
The Hong Kong strain of Influenza A virus used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-544). 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 2000 RPM for 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 (AppTec Lot VML-F53) was removed, thawed and refrigerated until use in the assay. The stock virus culture contained 1%fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Influenza virus on Rhesus monkey kidney cells.

2. Test Cell Cultures
Rhesus monkey kidney (RMK) cells were obtained from ViroMed Laboratories, Inc., Minneapolis, MN, Cell Culture Division. Cultures were maintained and used as monolayers in disposable tissue culture labware. On the day of testing, cells were observed as having proper cell integrity and therefore, were acceptable for use in this study.

3. Test Medium
Test medium used in this study was Eagles minimal essential medium (E-MEM) supplemented with 1% heat-inactivated fetal bovine serum (FBS), 10 µg/mL gentamicin, 100 units/mL penicillin, and 2.5 µg/mL Fungizone.

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

NUMBER OF DILUTIONS AND CULTURES FOR VIRUCIDAL EFFICACY STUDY			
Test or Control Group	**Dilutions Assayed (log_{10})**	**Cultures per dilution**	**Total Cultures**
Cell Control	N/A	4	4/group
Dried Virus Control (Group A)	-1,-2,-3,-4,-5,-6,-7,-8	4	32
Sample lot #1 + virus (Group B)	-1,-2,-3,-4,-5,-6,-7,-8	4	32
Sample lot #2 + virus (Group B)	-1,-2,-3,-4,-5,-6,-7,-8	4	32
Cytotoxicity of lot #1 (Group C)	-1,-2,-3,-4,-5,-6,-7,-8	4	32
Cytotoxicity of lot #2 (Group C)	-1,-2,-3,-4,-5,-6,-7,-8	4	32
Non-Virucidal level - lot #1 (Group D)	-1,-2,-3,-4,-5,-6,-7,-8	4	32
Non-Virucidal level - lot #2 (Group D)	-1,-2,-3,-4,-5,-6,-7,-8	4	32

METHODS

1. <u>Preparation of Test Substance</u>
AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE was used, undiluted, as received from the Sponsor. The test substance was in solution as determined by visual observation.

2. <u>Preparation of Virus Films</u>
Films of virus were prepared by spreading 0.2 mL of virus inoculum uniformly over the bottoms of three separate 100 X 15mm sterile glass petri dishes. The virus films were dried at 20.1°C in a relative humidity of 46% until visibly dry (20 minutes).

3. <u>Sephadex Gel Filtration</u>
To reduce the cytotoxic level of the virus-disinfectant mixture prior to assay of virus and/or to reduce the virucidal level of the disinfectant, virus was separated from disinfectant by filtration through Sephadex gel. Columns of Sephadex LH-20-100 were equilibrated with phosphate buffered saline containing 1% albumin, centrifuged for three minutes to clear the void volume, loaded with 2.0 mL of virus-disinfectant mixture and immediately passed through the column utilizing the syringe plunger.

4. <u>Treatment of Virus Films with Test Substance</u> (GROUP B, TABLE 1)
For each lot of disinfectant, separate dried virus films were exposed to 2.0 mL of the use dilution for 10 minutes at room temperature (22°C). Following the exposure time, the plates were scraped with a cell scraper to resuspend the contents of the plate and the virus-disinfectant mixture was immediately passed through a Sephadex column utilizing the syringe plunger in order to detoxify the mixture. The filtrate ([illegible] dilution) was then titered by serial dilution and assayed for infectivity.

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5. Treatment of Virus Control Films (GROUP A, TABLE 1)
A virus film was prepared as previously described (paragraph 2). The control film was exposed to 2.0 mL of test medium for the same amount of time as the test film was exposed to the disinfectant. The virus was then scraped and passed through a Sephadex column in the same manner as the test virus and the filtrate (10^{-1} dilution) was then titered by serial dilution and assayed for infectivity (paragraph 4).

6. Cytotoxicity Assay (GROUP C, TABLE 2)
A 2.0 mL aliquot of the use dilution of each lot of the disinfectant was filtered through a Sephadex column and the filtrate was diluted serially in medium and inoculated into RMK cell cultures. Cytotoxicity of the RMK cell cultures was scored at the same time as the virus-disinfectant and virus control cultures.

7. Assay of Non-Virucidal Level of Test Substance (GROUP D, TABLE 3)
Each dilution of the Sephadex-filtered disinfectant (disinfectant control for cytotoxicity assay) was mixed with an aliquot of low titer stock virus, and the resulting mixtures of dilutions were assayed for infectivity in order to determine the dilution(s) of disinfectant at which virucidal activity, if any, was retained. Dilutions that showed virucidal activity were not considered in determining the reduction in infectivity by the test substance.

8. Infectivity Assays
The RMK cell line, which exhibits CPE in the presence of Influenza A virus, 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. Cultures were incubated at 36-38°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures were scored periodically for seven days for the absence or presence of CPE, cytotoxicity, and for viability.

9. Statistical Methods: N/A

PROTOCOL CHANGES

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

Protocol Deviations: No protocol deviations occurred during this study.

DATA ANALYSIS

Calculation of Titers

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.

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

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STUDY RETENTION

Record Retention

All of the original raw data developed exclusively for this study shall be archived at AppTec Laboratory Services, 2540 Executive Drive, St. Paul, MN 55120. 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, E1053-97.
2. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November 1982.
3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.
4. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Schmidt, N.J. and Emmons, R.W. editors. Sixth edition, 1989. p. 18-20.
5. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.

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STUDY RESULTS

Results of tests with two lots of AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE (LOT# 2001.042.001 and LOT# 2001.005.001) exposed to Influenza A virus for 10 minutes are shown in Tables 1-3. All cell controls were negative for test virus infectivity. The titer of the dried virus control was 5.25 log_{10}. Following exposure, test virus infectivity was not detected in the virus-test substance mixture for either lot at any dilution tested ($\leq$0.5 log_{10}). Test substance cytotoxicity was not observed in either lot an any dilution tested ($\leq$0.5 log_{10}). The neutralization control (non-virucidal level of the test substance) indicates that the test substance was neutralized at $\leq$0.5 log_{10} for both lots. Taking the cytotoxicity and neutralization control results into consideration, the reduction in viral titer was $\geq$4.75 log_{10} for both lots.

STUDY CONCLUSION

Under the conditions of this investigation, in the presence of a 1% fetal bovine serum soil load, AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE (LOT# 2001.042.001 and LOT# 2001.005.001), ready to use, demonstrated complete inactivation of Influenza A virus as required by the U.S. EPA for virucidal label claims.

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

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TABLE 1: **Effects of AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE (LOT# 2001.042.001 and LOT# 2001.005.001) Following a 10 Minute Exposure to Influenza A virus Dried on an Inanimate Surface**

Dilution	Dried Virus Control (GROUP A)	Influenza A virus + LOT# 2001.042.001 (GROUP B)	Influenza A virus + LOT# 2001.005.001 (GROUP B)
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	+ + + +	0 0 0 0	0 0 0 0
10^{-2}	+ + + +	0 0 0 0	0 0 0 0
10^{-3}	+ + + +	0 0 0 0	0 0 0 0
10^{-4}	+ + + +	0 0 0 0	0 0 0 0
10^{-5}	+ + + 0	0 0 0 0	0 0 0 0
10^{-6}	0 0 0 0	0 0 0 0	0 0 0 0
10^{-7}	0 0 0 0	0 0 0 0	0 0 0 0
10^{-8}	0 0 0 0	0 0 0 0	0 0 0 0
$TCID_{50}$/0.1 mL	$10^{5.25}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$

TABLE 2: **Cytotoxicity of AXEN (EPA REG# 72977-2), THE 30 PPM USE DILUTION OF AXENOHL (EPA REG# 72977-1), A 2400 PPM CONCENTRATE on RMK Cell Cultures**

Dilution	Cytotoxicity Control LOT# 2001.042.001 (GROUP C)	Cytotoxicity Control LOT# 2001.005.001 (GROUP C)
Cell Control	0 0 0 0	0 0 0 0
10^{-1}	0 0 0 0	0 0 0 0
10^{-2}	0 0 0 0	0 0 0 0
10^{-3}	0 0 0 0	0 0 0 0
10^{-4}	0 0 0 0	0 0 0 0
10^{-5}	0 0 0 0	0 0 0 0
10^{-6}	0 0 0 0	0 0 0 0
10^{-7}	0 0 0 0	0 0 0 0
10^{-8}	0 0 0 0	0 0 0 0
TCD_{50}/0.1 mL	$\leq 10^{0.5}$	$\leq 10^{0.5}$

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present

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TABLE 3: Non-Virucidal Level of Test Substance (Neutralization Control)

Dilution	Test Virus + Cytotoxicity Control LOT# 2001.042.001 (GROUP D)	Test Virus + Cytotoxicity Control LOT# 2001.005.001 (GROUP D)
Cell Control	0 0 0 0	0 0 0 0
10^{-1}	+ + + +	+ + + +
10^{-2}	+ + + +	+ + + +
10^{-3}	+ + + +	+ + + +
10^{-4}	+ + + +	+ + + +
10^{-5}	+ + + +	+ + + +
10^{-6}	+ + + +	+ + + +
10^{-7}	+ + + +	+ + + +
10^{-8}	+ + + +	+ + + +

(+) = Positive for the presence of test virus after low titer stock virus added (neutralization control)
(0) = No test virus recovered and/or no cytotoxicity present

Results of the non-virucidal level control indicate that the test substance was neutralized at $TCID_{50}$ of $\leq 0.5 \log_{10}$ for both lots.

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