**Final Report**

**Quantitative surface test for the evaluation of fungicidal and yeasticidal activity of Hipochlorus acid in medical area on non-porous surfaces without mechanical action**

**Guideline(s)**

Series OECD on GLP

OECD n° 286 GIVIMP

UNI EN 17387

**Study Director**

Francesca Turra

**Date**

August 2024

|  |  |
| --- | --- |
| **Test Facility** | **Sponsor** |
| Renolab S.r.l. Via XXV Aprile, 19 I-40016 S. Giorgio di Piano (BO) Italy | Ahsitech Spain  Paseo de La Habana 187 |

**Test item:**  Hipochlorus acid

**Study code**: 24366-01M

**Statement of Confidentiality**

This report contains confidential and proprietary information of the sponsor which must not be disclosed to anyone except the employees of this company or to persons authorised by law or judicial judgement without the expressed and written approval of the sponsor.

Statement of Compliance with the Principles of  
Good Laboratory Practice

The study described in this report was conducted in compliance with the most recent edition of:

* Law Decree 2nd March 2007 N° 50 – Actuation of Directives 2004/9/EC and 2004/10/EC concerning the inspection and verification of Good Laboratory Practice (GLP) and aligning of laws, regulations and administrative provisions related to the application of the Principles of Good Laboratory Practice and to the control of their application in chemicals tests.
* The OECD Principles of Good Laboratory Practice.

The Italian requirements are based on the OECD Principles of GLP which are accepted by regulatory authorities throughout the European Community, the United States of America (FDA and EPA) and Japan (MHW, MAFF and METI) on the basis of intergovernmental agreements.

This report fully and accurately reflects the procedure used and data generated.

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Study Director Date

Statement of Quality Assurance Unit

|  |  |
| --- | --- |
| Study code: | 24366-01M |
| Study title: | Quantitative surface test for the evaluation of fungicidal and yeasticidal activity of Hipochlorus acid in medical area on non-porous surfaces without mechanical action |

The study plan was verified and then the experimental phase, raw data and a draft of the final report of this study were audited by the Quality Assurance in compliance with the OECD Guidelines and to Renolab’s Standard Operating Procedures.

Audit dates are given below:

| **Phase or document inspected** | **Date of audit/ verification** | **Date of report to** | | | |
| --- | --- | --- | --- | --- | --- |
| **Principal Investigator** | **Test site Management** | **Study Director** | **Test Facility Management** |
| Study plan: | June 14th 2024 | n.a. | n.a. | n.a. | n.a. |
| Experimental phase: | July 22nd 2024 | n.a. | n.a. | July 22nd 2024 | July 22nd 2024 |
| Raw data & draft report: | August 13th 2024 | n.a. | n.a. | August 13th 2024 | August 13th 2024 |

The final report corresponds to the raw data.

The signature on the present document indicates that the final report in its definitive version has been

checked.

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Quality Assurance Date

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# Summary

The fungicidal and yeasticidal activity of the test item “Hipochlorus acid” was *in vitro* tested for medical useson reference microorganisms selected by the Sponsor according to EN 17387, series of OECD on GLP and OECD n° 286 GIVIMP.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Test** | **Method** | **Microorganism** | **Product conc.** | **Conditions** | **Result (log)** |
| Efficacy test | UNI EN 17387 | *Candida Albicans* | 97% w/w | 30 seconds, room temperature | 2.66 |
| Efficacy test | UNI EN 17387 | *Aspergillus Brasiliensis* | 97% w/w | 30 seconds, room temperature | 1.06 |

# Time Schedule

| Study plan authorisation: | June 14th, 2024 |
| --- | --- |
| Start of experimental phase: | July 8th, 2024 |
| End of experimental phase: | July 29th, 2024 |
| Draft report: | August 26th 2024 |
| Study completion date: |  |
|  | |

# Study Objective

The aim of the current study is to evaluate whether the test item *Hipoclorous acid* possesses fungicidal and yeasticidal activity through an in vitro surface test without mechanical action in medical area.

The information on the test item was provided by the Sponsor.

# Material (s)

## Test Item

|  |  |
| --- | --- |
| Name product: | Hipochlorus acid |
| Appearance of the product: | Liquid solution with no color |
| Manufacturer: | Ahsitech Spain |
| Active ingredient(s)  common name: | HOCL (hypochlorus acid) |
| CAS Number of a.i.(s): | 7790-92-3 |
| Nominal content: | 400 ppm |
| Physical state: | Liquid solution |
| Batch number: | 1 |
| Renolab code: | 24366 |
| Manufacturing date: | 29/05/2024 |
| Expiry date: | 29/05/2026 |
| Safety precautions: | See SDS |
| Purity and composition: | All specifications about purity and composition of the test item are provided by the sponsor |

The Test item is provided by the Sponsor together with MSDS, the test item characterisation is the responsibility of the Sponsor

# Equipment

|  |  |  |  |
| --- | --- | --- | --- |
| **ID code** | **Equipment name** | **Brand** | **Model** |
| SA 308/00 | Technical balance | Kern | PCB 6000-1 |
| SA 188/00 | Autoclave | VWR | Vapour Line Eco |
| SA 360/00 | Automatic pipette P200 | Biosigma | HPpette Plus |
| SA 361/00 | Automatic pipette P1000 | Biosigma | HPpette Plus |
| SA 348/00 | Stopwatch | Houdian | NA |
| SA 341/03 | Datalogger | Marconi | LoRA SPYT1 |
| SA 350/00 | Laminar flow cabinet | Faster | Safe Fast Classic |
| SA 300/00 | Pipette filler motorized | VWR | Powerpette plus turquoise |
| SA 299/00 | Vortex | LLG Labware | uniTEXER, |
| SA 351/00 | Water bath | VWR | 18L 230V |
| SA 246/00 | Ultra-low temperature freezer | ARCTIKO | ULTF 420 |
| SA 341/30 | Datalogger | Marconi | LoRA SPYT3 |
| SA 236/00 | Incubator | Binder | KBF-S 240 |
| SA 136/00 | Datalogger | Escort | MU-IN-D-8-L |
| SA 195/00 | Microscope | Optech | Biostar |
| N.A. | Sterile steel discs 2cm | N.A. | N.A. |
| N.A. | Sterile glass beads | N.A. | N.A. |
| N.A. | General laboratory sterile plasticware and glassware | N.A. | N.A. |

## Reagents

| **Reagent name** | **Manufacturer** | **Internal Code** |
| --- | --- | --- |
| Distilled water | Aquaplus | N.A. |
| Malt Extract Agar (MEA) | Scharlab | 362 |
| Peptone from casein (tryptone) | Scharlab | 286 |
| Sodium chloride (NaCl) | Scharlab | 111 |
| Tween 80 | Scharlab | 395 |
| Sodium thiosulphate | Sigma Aldrich | 510 |
| L-alfa-Phosphatidylcholine | Sigma-Aldrich | 523 |
| Saponin | VWR | 364 |
| Bovine serum albumin Fraction V | Liofilchem | 415 |
| Sodium dodecyl sulfate | Sigma-Adrich | 380 |
| L-Histidine | VWR | 411 |

# Methods of Analysis

## Test organisms and experimental conditions

The test involved the following microorganisms in the following conditions:

| **CR** | **Microorganism** | **ATCC Code** | **Biosafety level** |
| --- | --- | --- | --- |
| CR019 | *Candida Albicans* | ATCC 10231 | 1 |
| CR020 | *Aspergillus Brasiliensis* | ATCC 16404 | 1 |

## Experimental conditions

The test consists of an *in-vitro* surface analysis (phase 2, step 2), where microbial suspension (N) mixed with an interfering substance was subjected to different concentrations of the test item for a defined time (t) at room temperature.

Chosen experimental conditions:

|  |  |
| --- | --- |
| **Test Item Concentration** | * 97% * 80% * 20% |
| **Test temperature** | Room temperature |
| **Contact time** | 60 seconds |
| **Test conditions** | Clean conditions (0.3 g/L bovine serum albumin) |

*The conditions of the test were given by the Sponsor.*

## Test organisms working culture

### Yeast working culture

Preparation of stock solutions was performed before the efficacy test in accordance with guideline EN 17387. Before running the test, the commercial culture was subcultured by streaking on to MEA plates and incubated at 30°C±1°C. After 48h, a second subculture from the first one was prepared and incubated for 40h-48h. This second subculture was directly used as working culture in the study experimental phase.

### Mould working culture

For *Aspergillus brasiliensis*, only the first subculture grown on MEA was necessary. It has been incubated for 9 days at 30°C ± 1°C and after incubation checked under microscope for the presence of at least 75% of spiny spores.

## Test suspension (N)

Test suspension was prepared from dilutions of the working culture with diluent (Tryptone 1g/L, NaCl 8.5 g/L, water up to 1L), adjusting the number of cells in order to obtain a suspension in the range between 1.5 x 108 to 5 x 108 cfu/ml.

## Test Item test solutions preparation

Test Item solutions were prepared by dilution with distilled water in order to achieve the desired test concentrations.

## Choice of the test method

The chosen neutralizer (Polysorbate 80, 60g/l + Saponin, 60g/l + L-alfa-Phosphatidylcholine, 9g/l + Sodium Thiosulphate, 10 g/l + histidine 4g/L + Sodium Dodecyl sulfate 4g/L), was validated during the analysis. It was capable of stopping the fungicidal and yeasticidal action of the product and avoid the toxic effect of the test item. For this reason the dilution neutralization method has been chosen.

### Test “Nd” – determination of yeasticidal and fungicidal concentrations

To evaluate yeasticidal and fungicidal activity, the test suspension (N) and the interfering substance were mixed and aliquoted onto test surface. After complete dryness, an aliquot of the test item solution was added onto surfaces for the chosen contact time (*t*) and temperature. At the end of the contact time, the test surface was neutralized in a suitable neutralizing agent and after neutralization time, Nd was diluted, spread onto MEA, incubated at 30°C ± 1°C and subsequently counted.

### Water control “Nc”

To validate the selected experimental condition and verify the absence of any lethal effect, a surface was inoculated with the mix of test organism suspension and interfering substance. After dryness, water was used instead of product for the chosen contact time (*t*) and temperature. After neutralization, an aliquot was diluted and spread, incubated and subsequently counted.

### Neutralizer control NC – verification of absence of toxicity of the neutralizer

To verify the absence of toxicity of the neutralizer, the neutralizer and water was mixed in a container. After 5 minutes the dried inoculated test surface was added to the container. The mixture was diluted, spread, incubated and subsequently counted.

### Method validation NT – dilution-neutralization validation

To validate the neutralization method, the neutralized was mixed with the highest product concentration used in the test, for the selected contact time and temperature. After *t,* an inoculated test surface was added in the container. The mixture was diluted, spread, incubated and subsequently counted.

## Incubation and counting

All the plates were incubated for 24 hours for *Candida albicans* and 48 hours for *Aspergillus brasiliensis*. The limits for counting colonies on agar plates for yeast are between 15 and 300 colonies, with an acceptable deviation of 10%, for dilution-neutralization method. Therefore, the limit accepted is 14-330.

The limits for counting colonies on agar plates for mould are between 15 and 160 colonies, with an acceptable deviation of 10%, for dilution-neutralization method. Therefore, the limit accepted is 14-165.

### Calculation of N

The number of cells in the test suspension (N), expressed as cfu/mL, was calculated taking into account two consecutive dilutions of the test suspension, according to the following formula:

Where:

c = sum of cfu counted per 1 mL of sample (dilution-neutralization method)

n1 = number of plates counted per mL of sample in the lower dilution

n2 = number of plates counted per mL of sample in the higher dilution

d = dilution factor of the lower dilution

For results calculated by weighted mean of two consecutive dilutions, the quotient of the means shall not be higher than 15 and not be lower than 5.

### Calculation of Nd and Nc

### For calculations of Nd, the number of survivors per test surface at the end of the contact time. Nc is the number of survivors per water control surface at the end of contact time, the following formula was used:

Where:

c = sum of cfu counted

n = number of plates counted per mL of sample

d = dilution factor taken into account

### Calculation of NC and NT

For calculations of NC and NT, the following formula was used:

Where:

c = sum of cfu counted

n = number of plates counted per mL of sample

d = dilution factor taken into account

### Calculation of Log Reduction (R)

The reduction R is expressed in decimal logarithm as the ratio between Nc and Nd:

At least one concentration per test shall demonstrate at least 4 log reduction and one concentration shall demonstrate a log reduction less than 4.

### Calculation and expression of results

Where

m, m’ are the two replicates at the lower dilution expressed in cfu

n, n’ are the two replicates at the higher dilution expressed in cfu

V is the volume of the inoculated into the plate expressed in ml

D is the lower dilution factor

Example:

If 168+ 213 cfu/plate are observed at the 10-6 dilution and 20+25 cfu/plate are observed at the 10-7 dilution, then *N* is:

In case not all replicas fall within the range, the average will take this into account:

* Only m and n fall in the range:
* Only m and m’ and n fall in the range
* Only m, n and n’ fall in the range
* Only m falls in the range

## Check of the limits of the test

For each test organism the calculated cfu/mL must be in the following ranges in order to consider the test valid:

|  |  |  |  |
| --- | --- | --- | --- |
| Microrganism | To be checked: | Results | Acceptability |
| *Candida albicans* | N between 6.57≤lgN≤7.10 | 6.87 | PASS |
| Nc shall be sufficiently high to demonstrate a 4 log reduction | 6.36 | PASS |
| NT-Nc is not greater than ± 0.3 log | ±0.02 | PASS |
| NC-Nc is not greater than ± 0.3 log | ±0.10 | PASS |
| Control of weighted means for N: quotient between 5 and 15 | 5.78 | PASS |
| Nts is less than 100 cfu/ml for active concentrations. For non-active concentration, Nts may be not countable | >100 | NOT APPLICABLE |

|  |  |  |  |
| --- | --- | --- | --- |
| Microrganism | To be checked: | Results | Acceptability |
| *Aspergillus brasiliensis* | N between 6.57≤lgN≤7.10 | 6.77 | PASS |
| Nc shall be sufficiently high to demonstrate a 4 log reduction | 6.33 | PASS |
| NT-Nc is not greater than ± 0.3 log | ±0.03 | PASS |
| NC-Nc is not greater than ± 0.3 log | ±0.04 | PASS |
| Control of weighted means for N: quotient between 5 and 15 | 8.77 | PASS |
| Nts is less than 100 cfu/ml for active concentrations. For non-active concentration, Nts may be not countable | >100 | NOT APPLICABLE |

# Summary results

The average values ​​of two replicates, concerning the validation of the method (NC, NC, NT) and the efficacy test (Nd) are reported below. Percentages of reduction of the viable colonies were calculated from mean values rounded to the nearest thousandths digit.

## Yeasticidal and fungicidal activity for *Candida albicans*

## Table 1: Average colony counting of *Candida albicans*

|  |  |  |  |
| --- | --- | --- | --- |
| **Test suspension titration (N)** | **Water control (Nc)** | **Neutralizer control (NC)** | **Validation method**  **Product. Conc. (97%) (NT)** |
| 6.87 | 6.36 | 6.26 | 6.34 |
| **Conc. of the product (%)** | **Log Nc** | **Log Nd** | **Log reduction (R)** |
| 97 | 6,36 | 3,70 | 2,66 |
| 80 | 6,36 | 4,15 | 2,21 |
| 20 | 6,36 | 4,68 | 1,68 |

Explanations:

Nd= is the number of survivors per test surface at the end of the contact time

R = reduction (lg *R* = lg*Nc* – lg*Nd*)

## Yeasticidal and fungicidal activity for *Aspergillus brasiliensis*

## Table 2: Average colony counting of *Aspergillus brasiliensis*

|  |  |  |  |
| --- | --- | --- | --- |
| **Test suspension titration (N)** | **Water control (Nc)** | **Neutralizer control (NC)** | **Validation method**  **Product. Conc. (97%) (NT)** |
| 6,77 | 6,33 | 6,37 | 6,36 |
| **Conc. of the product (%)** | **Log Nc** | **Log Nd** | **Log reduction (R)** |
| 97 | 6,33 | 5,27 | 1,06 |
| 80 | 6,33 | 5,46 | 0,88 |
| 20 | 6,33 | >5,52 | <0,81 |

Explanations:

Nd= is the number of survivors per test surface at the end of the contact time

R = reduction (lg *R* = lg*Nc* – lg*Nd*)

# Amendment to the study plan

|  |  |  |  |
| --- | --- | --- | --- |
| n. | Date | Change description | Impact |
| 1 | 22nd July 2024 | Error in writing the study plan. The contact time has been changed from 30 seconds to 60 seconds as per the sponsor's request. | None |

# Deviations from the Study Plan

|  |  |  |  |
| --- | --- | --- | --- |
| n. | Phase / trial | Deviation description / reason | Impact |
| N.A. | N.A. | N.A. | N.A. |

# Conclusions

The method of analysis provided in this study, aimed to demonstrate the yeasticidal and fungicidal action of the test item in accordance with UNI EN 17387 (September 2021). All controls and validations were within the basic limits but none of the tested concentration demonstrated a ≥ 4 lg reduction. No precipitates were observed during the test procedure (test mixtures were homogeneous). The experimental conditions were selected by the Sponsor.

According to EN 17387 the product ‘Hipoclorous acid’ (batch 1) **does not possess** fungicidal and yeasticidal activity in the medical area when used at 97 % w/w with room temperature against the specified strains of reference: *Candida albicans ATCC 10231 and Aspergillus brasiliensis ATCC 16404*

# Archiving

For the period demanded by the principles of GLP the following documents and materials will be archived:

* Study plan, raw data and the final report (10 years).
* A sample of the test item (1 year).
* All documentation generated by the Quality Assurance (10 years).

All materials and documents will be stored in the archives of the test facility Renolab S.r.l. The premises for storing of the documents and materials meet the principles of Good Laboratory Practice in the organisation of the test facility. At the end of the archiving period study-specific data or material will not be disposed of without the prior written consent of the Sponsor’s Representative.

# References

UNI EN 17387:2021 Chemical disinfectants and antiseptics – Quantitative surface test for the evaluation of yeasticidal and/or fungicidal activity of chemical disinfectants and antiseptics used in the medical area on non-porous surfaces without mechanical action – Test method and requirements (phase 2, step 2).

OECD Series on Testing and Assessment

OECD N. 286: Guidance Document on Good in Vitro Method Practices (GIVIMP)

OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring

OECD N. 14: The Application of the Principles of GLP to in vitro Studies

OECD N.19: Advisory Document of the Working Group on Good Laboratory Practice on the Management, Characterisation and Use of Test Items

# Distribution

|  |  |  |  |
| --- | --- | --- | --- |
|  | Study Plan | Raw Data | Final Report |
| Test Facility: | 1 original | 1 original | 1 original |
| Sponsor: | 1 pdf copy | none | 1 pdf copy |

# Appendix

## GLP certificate of test facility

Immagine che contiene testo, lettera, menu, documento

Descrizione generata automaticamente