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INVESTIGATION, DEVELOPMENT AND VALIDATION OF THE DISINFECTANT USED IN STERILE PHARMACEUTICAL MANUFACTURING FACILITY

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ABSTRACT

Objective: Cleaning and disinfection of surfaces are crucial process for maintaining the integrity of pharmaceutical manufacturing operations. One of the bigger challenge faced by pharmaceutical manufacturing is with the choice of disinfectants, for ensuring that the disinfectants selected are appropriate for use and that the affectivity of the disinfectants are periodically assessed. For both sterile and non-sterile pharmaceutical product, the severity of the effects of microbial contamination is very much a function of the nature of the contaminated product, its intended use and the number of contaminants. In this study disinfectant (ACITAR) was assessed and validated for its use in sterile manufacturing facility. The validation was based upon the regulation guidelines provided by W.H.O, USP and other guidelines and the disinfectants were proved to be effective in various condition. **Methods:** Three different methods were used to test the efficacy of the disinfectant, they are-membrane filtration and direct inoculation method, swab analysis method and agar diffusion or ditch plate method. **Results:** Tests proved that the disinfectant was active against the standard bacteria and fungi. Acitar 5% solution showed its action within 10 minutes in both membrane filtration and swab analysis. Agar diffusion method showed a perfect zone of inhibition for the respective disinfectant. **Conclusion:** The validation of disinfectant was done in accordance to the

procedures given in I.P and W.H.O guidelines. The results from different methods were promising and the process was validated, a protocol was generated and SOP was prepared for the further assessment of disinfectant.

KEYWORDS: Validation, Disinfectant, Antimicrobial agent, Membrane filtration, Microbial Contamination.

Fig. 1: Image of the disinfectant.

- **INTRODUCTION**

Validation is necessary part of quality assurance plan and is fundamental to an efficient production operation. This concept pioneered by 2 FDA officials – **TED BYERS and BUD LOFTUS in 1970.** The U.S. Food and Drug Administration (FDA) has proposed guidelines with the following definition for validation.[1,2]

"Process validation is the concept of establishing documented evidence which provides a high degree of assurance that a individual process (such as the manufacture of pharmaceutical dosage forms) will consistently produce a commodity meeting its predetermined specifications and quality characteristics."[6,7]

According to the FDA, assurance of quality of the product is obtained from precise and fundamental attention to a number of important factors, including;

1. Selection of quality components and materials.
2. Adequate product and process design.
3. Control of process through in-process and end-process testing.

Ample progress has been made in determining and understanding the mechanisms of the antimicrobial action of antiseptics and disinfectants. By contradiction, studies on their modes of action against fungi, viruses and protozoa have been rather sparse. Moreover, less is known about the means whereby these agents pacify prions. Whatsoever the type of microbial organism (or entity), it is probable that there is a common array of events. This can be envisaged as interaction of the antiseptic, sterilant or disinfectant along with the cell surface followed by penetration into the cell and action at the target site(s). The nature and composition of the surface differ from one cell type (or entity) to another but can also modify as a result of changes in the environment.[6,7,8]

Generally disinfectants are "cidal" in that they wipe-out the susceptible probable pathogenic agents. The choice of a disinfectant should be established on the action the disinfectant is expected to act, not necessarily on a sales pitch or on what you have always used. Ideally, a disinfectant should be non-irritating, nontoxic, broad spectrum (eliminates bacteria, viruses, protozoa, fungi and spores), noncorrosive and inexpensive. Decisions on the choice should combine effectiveness against the potential pathogenic agent, safety to individuals, effect on machinery, the environment, and cost.[8,19]

Need[6,17,19]

1. To reduce the overall bio-burden of production and other classified areas.
2. To destroy the microbes present in critical area.
3. Prevent the entry of micro-organism into the facilities.
4. To develop a sense of responsibility in the workers towards maintaining sterile environment.
5. Prevent dissemination of microbes throughout the facility and eliminate the buildup of pyrogen.
6. To decontaminate the microbial build up occurred by spillage.
7. To make the product free from any types of microbes.

- **MATERIALS AND METHODS**

✓ **Test Materials**

Hot air oven(Modern Industrial Co-operation), Ultra Sonicator(Lab Hosp Corporation), Laminar Air Flow Unit(Kleanzone Systems India Private Limited), Colony Counter(Lab Hosp Corporation), Dry Bath(Neolab Instruments), Autoclave(Modi Enterprises Corporation), Weighing Balance(Mettler Toledo), Heating Mantle(Bio Technics India),

Microscope, Pump, Micropipettes, Microtips, Petri-Plates, Glass Bottles, Filtration Assembly, Volumetric Flask, Incubator.

✓ **Bacterial Strains**

S. aureus (6538P), E. coli (4157), P. aeruginosa (15442), B. subtilis (6633), S.abony (6017), A. brasiliensis (16404), C. albicans (10231).

✓ **Bacterial Growth Media**

Soyabean Casein Digest Agar (SCDA), Soyabean Casein Digest Medium, Sabouraud Dextrose Agar with Cholramphenicol (SDA), Cetrmide Agar, Pseudomonas Agar Medium for Detection of Pyocyanin (PPA), Pseudomonas Agar Medium for Detection of fluroscein (PPF), Mannitol Salt Agar (MSA), MacConkey Agar (MA), Fluid Thiogylcollate Medium (FTM), Xylose-Lysine Deoxycholate Agar (XLDA), Wilson and Blair's BBS Agar (WBBS), Sterile Cotton Swabs in Screw capped.

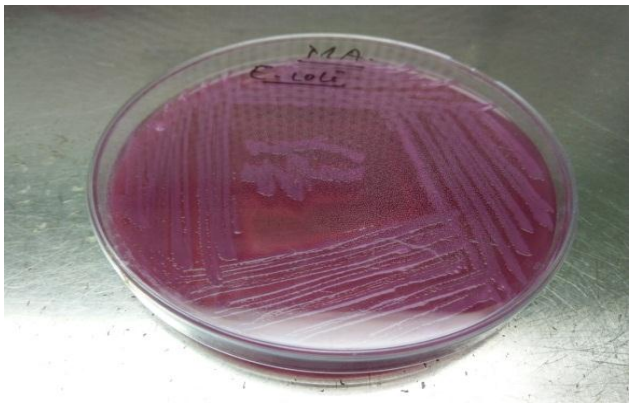


Fig. 2- MacConkeys Agar streaked with standard strain of *E. coli (4157)* which yields pink coloured colonies after growth.

- **PROCEDURE AND OBSERVATIONS**

1. Microbiological testing procedure[1,3,4,5]

METHOD USED: LOG REDUCTION OR SUSPENSION METHOD

A) Procedures for preparation of Bacterial and Fungal Suspension

1. Take 24 hours grown bacterial cultures of *S. aureus (6538P), E. coli (4157), P. aeruginosa (15442), B. subtilis (6633), S.abony (6017), A. brasiliensis (16404)* and *C. albicans (10231).*
2. Add 3ml of 0.9% sterile saline. This corresponds to 10° dilution.

B) Procedure for Serial Dilution of Bacterial and Fungal cultures

1. Aseptically transfer 1 ml of above prepared bacterial culture suspension of E. coil (ATCC 4157) to the vial containing 9ml of sterile saline. This corresponds to 10^{-1} dilution.
2. Aseptically mix the content well. Transfer 1ml of the dilution 10^{-1} to the vial containing 9ml of sterile saline. This corresponds to 10^{-1} dilution.
3. Continue the procedure to make further dilution viz. 10-3, 10-4 up to 10-8
3. Repeat steps from 1 to 3 for the serial dilutions remaining bacterial cultures, viz *S. aureus (6538P), P. aeruginosa (15442), B. subtilis(6633), S.abony(6017), A. brasiliensis(16404)* and *C. albicans(10231).*

C) Test Procedures (Bactericidal and Fungicidal Activity)

1. Place 2.5 ml of undiluted disinfectant into sterile test tubes separately for each of the dilution of *E. coil (ATCC 4157)* viz. 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}cultures.
2. Suspend 0.25ml of the suspension of each dilution viz. 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} of E. coil (ATCC 4157) individually in each tube containing undiluted disinfectant (ACITAR).
3. After specified time intervals viz. 10 mins, 20 mins, 30 mins, 40 mins and 50 mins inoculate 0.25 ml of the test suspension (micro-organism + disinfectant) from each vial of step 2 to individual vial containing 20 ml of molten Sterile Plate Count Agar (PCA) media.
4. Mix the media and culture properly, pour into individual sterile petri-plates and allow it to solidify completely.
5. Repeat step 1-4 for remaining bacterial cultures viz. *S. aureus (6538P), E. coli (4157), P. aeruginosa (15442), B. subtilis (6633), S.abony (6017).*
6. Repeat steps 1 to 2 for fungal cultures viz. *C. albicans (ATCC 10231) and A. Niger (ATCC 16404).*
7. After specified time intervals viz. 10 mins, 20 mins, 30 mins, 40 mins and 50 mins inoculate 0.25 ml of the test suspension (micro-organism + disinfectant) from each vial of step 6 to individual vial containing 20 ml of molten Sterile Dextrose agar (SDA) media.
8. Mix the media and culture properly, aseptically pour into individual sterile petri plates and allow it to solidify completely.
9. Incubate the plates viz. 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, $10^{-7}$of *S. aureus (6538P), E. coli (4157), P. aeruginosa (15442), B. subtilis (6633), S.abony (6017)* at 37°C for 72 hours.

10. Incubate the plate viz. 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} of. *C. albicans* (ATCC 10231) at (25-30) ° C for 120 hours and the plates of *A. brasiliensis* (ATCC 16404) at (25-30) °C for 168 hours.
11. Repeat step 1 to 10 using 1% v/v, 2% v/v, 3% v/v, 4% v/v, 5% v/v, 6% v/v & 7% v/v dilutions of ACITAR.
12. Observe the plates to check the colony count.
13. For bacterial cultures run the positive control by individually inoculating 0.25ml of each dilution viz 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, $10^{-7}$of *E. coli* (ATCC 4157), *S. aureus* (ATCC 6538P), *P. aeruginosa* (ATCC 15442), *B. subtilis* (ATCC 6633), *S.abony(6017)* directly in 2.5ml of 0.9% sterile saline instead of the disinfectant and further inoculating 0.25ml of this in the vials containing 20ml of sterile Plate Count Agar (PCA) media. Mix the media and culture properly. Aseptically pour into individual sterile petriplates and allow it to solidify completely.
14. For fungal cultures run the Positive Control by individually inoculating 0.25ml of each dilutions viz. 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, of. *C. albicans* (ATCC 10231), and *A. brasiliensis* (ATCC 16404) directly in 2.5ml of 0.9% Sterile saline instead of the disinfectant and further inoculating 0.25ml of this in the vials containing 20ml of Sabouraud Dextrose Agar (SDA) media. Aseptically mix the media and culture properly and pour into individual sterile petriplates and allow it to solidify completely.
15. Incubate the plates of bacterial culture as mentioned in the step 9. And fungal cultures mentioned in step 10.
16. Observe the plates to check the colony count.
17. Run the Negative Control by inoculating 250ul of 0.9% Sterile Saline used for the test in 20ml of Sterile Plate Count Agar (PCA) media and 20ml of Sterile Sabouraud Dextrose Agar (SDA) media.
18. Mix the contents uniformly and pour into individual sterile petridishes and allow it to solidify completely.
19. Incubate the Plate Count Agar of Negative Control at 37^{0}C for 72 hours, the Potato Dextrose Agar plate of Negative Control at (25-30) ^{0}C for 168 hours.
20. Observe the Negative Controls for any Bacterial and Fungal Contamination.

$$\text{Viable Count} = \frac{\text{Average number of colonies X Dilution Factor}}{\text{Amount of Inoculum (0.25ml)}}$$

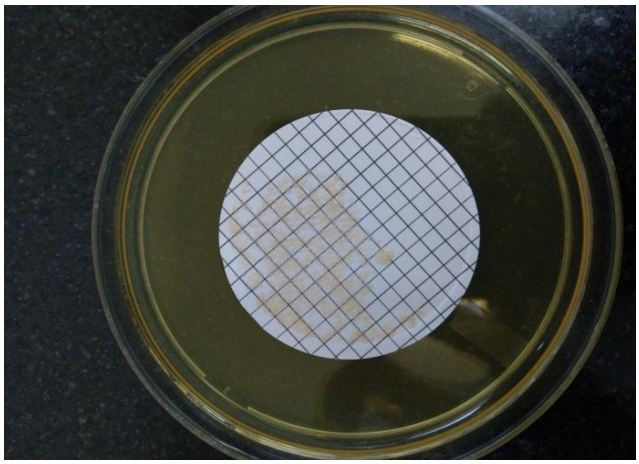


Fig. 3: Membrane filter placed on SCDA plate with colonies of microbes grown on it.

POSITIVE CONTROL

Title: Positive control (without disinfectant)

Table no 1: Results without disinfectant after incubation at 37°c for 72 hrs.

Micro-Organisms	ATCC NO	Dilutions								Viable Count(Cfu/Ml)
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
E. coli	4157	M	M	UC	UC	UC	91	30	03	9.21×10^{7}
S. aureus	6538P	M	M	UC	UC	UC	104	32	03	9.65×10^{7}
P. aeruginosa	15442	M	M	UC	UC	UC	112	40	02	9.49×10^{7}
B. subtilis	6633	M	M	UC	UC	UC	95	38	02	9.00×10^{7}
S. abony	6017	M	M	UC	UC	UC	101	29	01	6.54×10^{7}

Table no 2: Result after incubation at 25°c - 30°c for 120 hrs.

Micro-Organisms	ATCC NO	Dilutions								Viable Count(Cfu/Ml)
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
C. albicans	10231	M	M	UC	UC	UC	80	24	03	8.04×10^{7}

Table no 3: Result after incubation at 25°c - 30°c for 168 hrs.

Micro-Organisms	ATCC NO	Dilutions								Viable Count(Cfu/Ml)
		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
A. brasiliensis	16404	M	M	UC	UC	UC	78	30	02	7.70×10^{7}

M=MATT GROWTH UC=UNCOUNTABLE CFU=COLONY FORMING UNIT

Title: Test with disinfectant with 10 mins contact time.

Table no 4: Results with disinfectant – ACITAR (test) with 10 mins contact time. Result after incubation at 37°c for 72 hrs.

Micro-Organism	ATCC NO.	Conc %V/V	Dilutions								Log Reduction/ Contact Time
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
E. coli	4157	1	M	UC	UC	UC	79	21	8	0	7 Log reduction/10 mins
		2	M	UC	UC	102	60	16	5	0	
		3	M	UC	114	86	43	11	0	0	
		4	UC	62	27	0	0	0	0	0	
		5	0	0	0	0	0	0	0	0	
S. aureus	6538P	1	M	UC	UC	141	98	45	11	0	7 Log reduction/10 mins
		2	M	UC	91	62	25	9	0	0	
		3	UC	84	58	14	8	0	0	0	
		4	UC	64	18	0	0	0	0	0	
		5	0	0	0	0	0	0	0	0	
P. aeruginosa	15442	1	M	UC	UC	160	113	76	27	0	7 Log reduction/10 mins
		2	M	UC	UC	124	98	69	22	0	
		3	M	UC	111	82	57	18	0	0	
		4	UC	71	34	0	0	0	0	0	
		5	0	0	0	0	0	0	0	0	
B. subtilis	6633	1	M	UC	UC	UC	97	49	13	0	7 Log reduction/10 mins
		2	M	UC	UC	117	84	42	11	0	
		3	M	UC	UC	82	35	6	0	0	
		4	UC	UC	62	25	3	0	0	0	
		5	0	0	0	0	0	0	0	0	
S. abony	6017	1	M	UC	UC	UC	84	43	17	0	7 Log reduction/10 mins
		2	M	UC	UC	101	68	23	11	0	
		3	M	UC	121	74	48	16	7	0	
		4	UC	82	20	0	0	0	0	0	
		5	0	0	0	0	0	0	0	0	

Table no 5: Result after incubation at 25°c - 30°c for 120 hrs.

Micro-Organism	ATCC NO.	Conc %V/V	Dilutions								Log Reduction/ Contact Time
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
C. albicans	10231	1	M	UC	UC	UC	75	28	3	0	7 Log reduction/10 mins
		2	M	UC	86	71	47	7	0	0	
		3	UC	UC	74	65	37	4	0	0	
		4	UC	47	16	0	0	0	0	0	
		5	0	0	0	0	0	0	0	0	

Table no 6: Result after incubation at 25°c - 30°c for 168 hrs.

Micro-Organism	ATCC NO.	Conc %V/V	Dilutions								Log Reduction/ Contact Time
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
A.brasiliensis	16404	1	M	UC	UC	UC	73	37	11	0	7 Log reduction/10 mins
		2	M	UC	123	92	64	28	9	0	
		3	UC	UC	117	86	52	17	6	0	
		4	UC	78	41	15	0	0	0	0	
		5	0	0	0	0	0	0	0	0	

As the above results shows that the growth of micro-organism had been ceased by 5% v/v Acitar solution, now we need to check the lower limit of the time required by ACITAR at 5% v/v to show its bactericidal and fungicidal effect.

Here the concentration has been fixed at 5% v/v but the activity of ACITAR (5% v/v) below 10 mins is needed to be checked to determine the end point.

Title: Test with disinfectant with 5 mins contact time.
Table no 7: Results with disinfectant – ACITAR (test) with 5 mins contact time. Result after incubation at 37°c for 72 hrs.

Micro-Organism	ATCC NO.	Conc %V/V	Dilutions								Log Reduction/ Contact Time
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
E. coli	4157	1	M	M	UC	UC	UC	84	29	4	-
		2	M	M	UC	UC	UC	89	38	6	
		3	M	M	UC	UC	UC	74	22	2	
		4	M	M	UC	UC	67	37	16	0	
		5	M	M	UC	UC	48	13	0	0	
S. aureus	6538P	1	M	M	UC	UC	UC	97	35	7	-
		2	M	M	UC	UC	112	86	28	3	
		3	M	M	UC	UC	72	24	14	0	
		4	M	M	UC	UC	63	20	11	0	
		5	M	M	UC	UC	59	15	0	0	
P. aeruginosa	15442	1	M	M	UC	UC	UC	97	48	8	-
		2	M	M	UC	UC	UC	83	42	6	
		3	M	M	UC	UC	86	38	16	0	
		4	M	M	UC	UC	78	34	14	0	
		5	M	M	UC	UC	54	12	0	0	
B. subtilis	6633	1	M	M	UC	UC	UC	95	47	4	-
		2	M	M	UC	UC	UC	88	39	2	
		3	M	M	UC	UC	75	32	12	0	
		4	M	M	UC	UC	64	28	09	0	
		5	M	M	UC	UC	63	21	0	0	
S. abony	6017	1	M	M	UC	UC	UC	102	36	5	-
		2	M	M	UC	UC	UC	98	32	3	
		3	M	M	UC	UC	85	41	12	0	
		4	M	M	UC	UC	78	35	06	0	
		5	M	M	UC	UC	64	25	0	0	

Table no 8: Result after incubation at 25°c - 30°c for 120 hrs.

Micro-Organism	ATCC NO.	Conc %V/V	Dilutions								Log Reduction Contact Time
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
C. albicans	10231	1	M	M	UC	UC	UC	128	37	3	-
		2	M	M	UC	UC	UC	120	34	2	
		3	M	M	UC	UC	119	82	27	0	
		4	M	M	UC	UC	94	79	23	0	
		5	M	M	UC	92	68	32	0	0	

Table no 9: Result after incubation at 25°c - 30°c for 168 hrs.

Micro-Organism	ATCC NO.	Conc %V/V	Dilutions								Log Reduction/ Contact Time
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
A.brasiliensis	16404	1	M	M	M	UC	UC	122	80	2	-
		2	M	M	M	UC	UC	106	70	1	
		3	M	M	M	UC	UC	97	15	0	
		4	M	M	UC	UC	124	72	18	0	
		5	M	M	UC	UC	57	8	0	0	

As the above results shows that the organisms are unaffected at a contact time of 5 minutes hence the time is been selected to be 10 minutes.

2. SWAB SAMPLING[10,11]

Swab (or wipe) analysis can be used to detect and identify organic and inorganic contaminants (dusts, pesticides, metals, spray drift, contaminant residues, etc.) on different surfaces present in the manufacturing facility. The approach is highly effective on smooth surfaces such as glass, metal (including pipes), painted surfaces, epoxy and smooth vegetation surfaces such as leaves. Swab sampling has limited effectiveness on surfaces that are rough and/or porous (e.g. timber and concrete).

Fig. 4: Image of the swab used to swab analysis method.

1. Select standard area/areas to swab (minimum of 10cm^2). Record the standard area.
2. Gloves must be worn before doing the swab analysis. The gloves should be clean and powder free.
3. The standard area on the surface to be wiped must be pre-marked. Care should be taken when pre-marking a surface. For example, pre-marking a surface with permanent marker and then using an organic solvent may lead to the permanent marker ink dissolving into the solvent and causing contamination. Alternatively, a pre- cut template adhered against the surface can be used.
4. Wet each swab with relevant solvent. Keep record of the solvent used.
5. Wipe the swab across the pre-marked surface from left to right using an appropriate pressure and hold the swab flat against the surface.
6. Continue the swabbing until the entire surface has been wiped (Figure 1a).
7. Re-wipe the surface again from top to bottom (Figure 1b).
8. Re-wipe the surface again from bottom left to top right (Figure 1c).
9. Re-wipe the surface again from top left to bottom right (Figure 1d).
10. Used swabs should be properly placed in labelled sampling containers appropriate for the storage of the analytes of interest (e.g. while washing a glass jar with organic solvents when pesticides are the contaminants of interest). Record the sample name, date, site, time and sampler's name in a notebook or equivalent.
11. If a template has been used, it must be cleaned appropriately, dried before using it at another site of interest.
12. A blank field must be taken by wetting the swab/s with the solvent and placing the swab in the jar. When only minute levels of contamination are anticipated like in class A, a larger surface area should be swabbed (up to 1m^2). More than one swab may be used, and swabs can be pooled for analysis. There is a huge possibility of matrix interferences and contamination using readily available materials and therefore their use is only recommended for urgent sampling conditions when only the presence or absence of a contaminant is analysed. All the solvents that are used to wet the swab or wipe must be of analytical grade. Organic solvents, such as isopropyl alcohol, acetone, ethanol, hexane etc. are generally used. Ultrapure water or sterile water is only an appropriate wetting solvent for inorganic dusts. You should contact your laboratory staff for advice on suitable solvents for the analytes of interest.

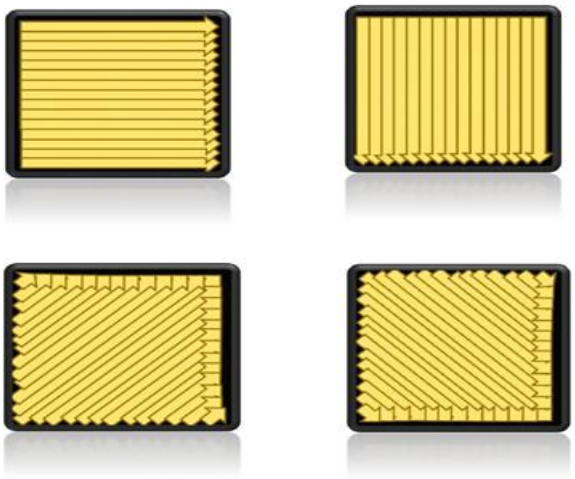


Fig. 5: Example of procedure for swab sampling, with black outline indicating the pre-marked area to be swabbed, yellow arrows indicating the direction for swiping. Swabbing starts as a) swab the marked area from left to right horizontally, b) swab the marked area from top to bottom vertically c) swab the marked area from bottom left to top right, and d) swab the marked area from top left to bottom right.

OBSERVATION TABLE

Table no 10: Result of swab analysis of different organisms using ACITAR 5% V/V solution.

Sr.No	Micro-Organism	Dilution	Results
1	*E. coli (*4157*)*	10^4	No Growth
2	*S. aureus (*6538P*)*	10^3	No Growth
3	*P. aeruginosa (*15442*)*	10^4	No Growth
4	*B. subtilis(*6633*)*	10^4	No Growth
5	*S.abony(6017)*	10^3	No Growth
6	*C. albicans(*10231*)*	10^3	No Growth
7	*A. brasiliensis(*16404*)*	10^3	No Growth

3. Agar Well Diffusion Method[6,12,15]

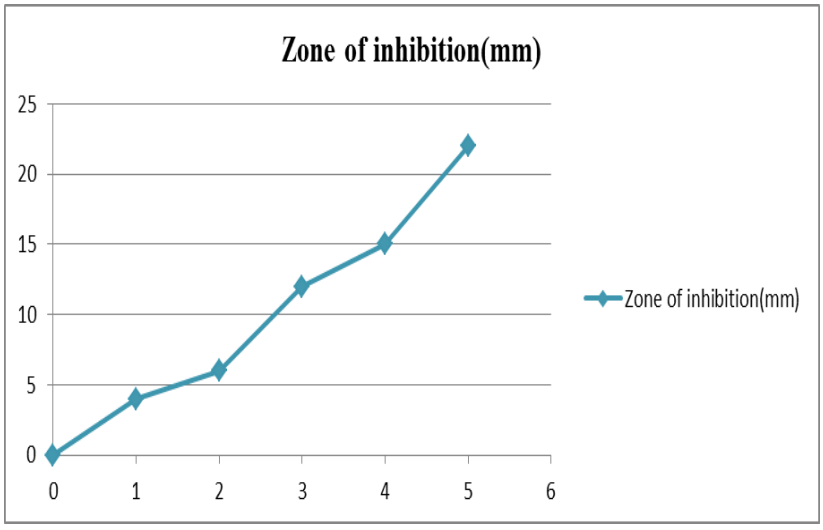
The method indicates susceptible of the challenged organism to the tested disinfectant by a clear zone of inhibits growth around the well that is bored in the agar medium. Upon contact with the agar surface, the disinfectant diffuses into the medium. The growth of the micro-organism is subdued until a critical concentration (comparable to the Minimal Inhibitory Concentration or MIC) is attained. The diameter of the resulting zone is considered proportional to the degree of susceptibility and allows to categorize the disinfectant into suitable class.

1. Prepare SCDA plates by preparing SCDA medium and aseptically pouring approximately 10-15 ml into sterile petri plates followed by solidifying at room temperature.
2. After solidification label all the plates with the name of media, preparation batch no and date of preparation.
3. Incubate the prepared plates at 30-35°C for 24-48 hrs. And check if there is any contamination. If contamination occurs then discard the plates.
4. After incubation, the specified concentration of micro-organisms to be challenged are spread over the surface
5. The wells are prepared by using the sterile cork borer or a tip
6. A volume (20–100 mL) of the antimicrobial agent at desired concentration is introduced into the well
7. Incubate the plates for 24hr. The antimicrobial agent diffuses through the agar medium and zone of inhibition is formed.
8. Measure the zone of inhibition.

Observation table

Table no 11: Result of the different concentration of ACITAR along with their zone of inhibition in millimetres.

Sr.No	Conc of the disinfectant(%V/V)	Zone of inhibition(mm)
1	0	0
2	1	4
3	2	6
4	3	12
5	4	15
6	5	22



Graph showing the results of concentration vs. zone of inhibition.

RESULTS

Table no 12: Result table of the final test showing the Log reduction value of the disinfectant.

Micro-Organisms (ATCC No. / NCIM No.)	Culture Dilution [Viable count]	Contact time (Mins)	10	20	30	Conclusion
E. coli (4157)	A= 9.21 x 10^7 log A= 7.964	CFU / 0.1 mL	0	0	0	Acitar 5%V/V solution gives >7 Log Reduction in 10 Mins
		Log of CFU (B)	0	0	0	
		Log reduction (A-B)	7.964	7.964	7.964	
S. aureus (6538P)	A= 9.65 x 10^7 log A= 7.984	CFU / 0.1 mL	0	0	0	Acitar 5%V/V solution gives >7 Log Reduction in 10 Mins
		Log of CFU (B)	0	0	0	
		Log reduction (A-B)	7.984	7.984	7.984	
S. abony (6017)	A= 6.54 x 10^7 log A= 7.8155	CFU / 0.1 mL	0	0	0	Acitar 5%V/V solution gives >7 Log Reduction in 10 Mins
		Log of CFU (B)	0	0	0	
		Log reduction (A-B)	7.8155	7.8155	7.8155	
B. subtilis (6633)	A= 9.00 x 10^7 log A= 7.9542	CFU / 0.1 mL	0	0	0	Acitar 5%V/V solution gives >7 Log Reduction in 10 Mins
		Log of CFU (B)	0	0	0	
		Log reduction (A-B)	7.9542	7.9542	7.9542	
P. aeruginosa (15442)	A= 9.49 x 10^7 log A= 7.9772	CFU / 0.1 mL	0	0	0	Acitar 5%V/V solution gives >7 Log Reduction in 10 Mins
		Log of CFU (B)	0	0	0	
		Log reduction (A-B)	7.9772	7.9772	7.9772	
A. brasiliensis (16404)	A= 7.70 x 10^7 log A= 7.886	CFU / 0.1 mL	0	0	0	Acitar 5%V/V solution gives >7 Log Reduction in 10 Mins
		Log of CFU (B)	0	0	0	
		Log reduction (A-B)	7.886	7.886	7.886	
C. albicans (10231)	A= 8.04 x 10^7 log A= 7.9052	CFU / 0.1 mL	0	0	0	Acitar 5%V/V solution gives >7 Log Reduction in 10 Mins
		Log of CFU (B)	0	0	0	
		Log reduction (A-B)	7.9052	7.9052	7.9052	

The above table shows the result about the action of the disinfectant i.e ACITAR on different micro-organisms. It is proved that ACITAR shows more than 7 log reductions on each of the micro-organism. Along with membrane filtration study the other studies like swab analysis and agar diffusion study also made it clearer that ACITAR could be effective against all the

standard bacteria. As the process is been validated a new SOP is prepared for the validation of the disinfectant that might be required in the near future.

An SOP was prepared after the entire validation of the disinfectant was performed and thereby the SOP will help for further assessment. The SOP is as follows:

❖ Sop for the validation of the disinfectant:

1. Objective

This document describes the testing procedure for the efficacy of different type of chemical disinfectant.

2. **Scope**

This document provides the procedure for validating the sanitizers or disinfectant and the sanitization procedure being followed in the manufacturing and the testing facilities in pharmaceuticals.

3. Reference Document

✓ SOP for microbiological culture media preparation

✓ Disinfectant efficacy test

4. Pre-requisites

Prior to conducting or executing the general validation protocol following things must be available;

4.1 Microbial Standard Culture

4.1.1 *Bacillus Subtilis(6633)*

4.1.2 *Escherichia coli(4157)*

4.1.3 *Staphylococcus aureus(6538P)*

4.1.4 *P. aeruginosa(15442)*

4.1.5 *S. abony(6017)*

4.1.6 *Candida albicans(10231)*

4.1.7 *Aspergillus brasiliensis (16404)*

4.1.8 Environment isolates

4.2 Disinfectants: All disinfectants used in the facility

4.3 Micropipettes

4.4 Sterilized tips and petri plates

5.0 Responsibility

The Responsibilities and the roles of Quality Control and Quality Assurance and Microbiology personnel involved in activities related to the validation process are defined below:

5.1 Quality Assurance (Validation)

5.1.1 Approval of protocol

5.2 Quality Control and Microbiology

5.2.1 Preparation of protocol

5.2.2 Review of protocol

5.2.3 Execution of protocol

6.0 Validation Method

Qualification Tests

A) Suspension Method (validation of sterilizer)

B) Surface spray or wipe method (validation of sanitization method)

6.1 Suspension Method

6.1.1 Objective

To establish the test concentration and the contact time suspension test is generally applied. The suspension test helps to estimate the in vitro bactericidal activity of the disinfectant under precise and controlled experimental conditions including

- Microbial strain
- Preparation of inoculum
- Volume of inoculum vs. Disinfectant
- Temperature
- Disinfectant Concentration and contact period
- Interfering substances

6.1.2 Procedure

6.1.2.1 Prepare the culture suspension from the original as per SOP for preparation of microbiological culture media.

6.1.2.2 Select the dilutions which yield 10^5 to 10^6 cells per ml.

6.1.2.3 Prepare 10 ml of test dilutions to be tested with sterile distilled water.

6.1.2.4 Vortex the test tube for 1minute.

6.1.2.5 Add 0.1 ml of any one culture containing 10^5 to 10^6 cells per ml into the test disinfectant with the decided concentration.

6.1.2.6 The final concentration of organisms shall be 10^4 to 10^5 cells per the tube. consider the preparation for 0 min.

6.1.2.7 Prepare like the same above for other three different time intervals

6.1.2.8 Give a contact time of 0 min, 5 mins, 10 mins, 15 mins, 20 mins and 30 mins.

6.1.2.9 After the specified contact period, filter the samples through a 0.45 m membrane filter and keep aside.

6.1.2.10 Give three washing of 100 ml each with 0.1% sterile peptone water / sterile water with constant stirring.

6.1.2.11 After filtration with the help of sterile forceps takes the membrane filter and place it on a Soybean casein digest agar.

6.1.2.12 Incubate the bacterial culture at 30 to 35^0 c for 24 to 48 hrs. and fungal cultures at 20 to 25^0c for 72 to 120 hrs.

6.1.2.13 After incubation counts the no. of colonies present on the membrane.

6.1.2.14 Note down the number of colonies in a register.

6.1.2.15 This will be observed count after the exposure.

6.1.2.16 Select the plates, which have least nil count.

6.1.2.17 Proceed in the same manner taking all the culture to be tested.

6.1.2.18 Contact time for the usage of the disinfectant will be set on the basis of result, which will have least count.

6.2 Surface spray / wipe method / Swab method.

6.2.1 Objective

To establish the effectiveness of the test concentration and the contact time generally applied the suspension test estimates the in vitro bactericidal activity of the disinfectant under precise experimental condition including;

- Microbial strain
- Preparation of inoculum
- Volume of inoculums vs. disinfectant
- Temperature
- Disinfectant concentration and contact period
- Interfering substances (i.e. Inorganic and organic matter) With the swab or wipe method the following surfaces shall be taken for validation

- Stainless steel(SS)
- Epoxy
- Panel
- PU Paint wall (Poly Urethane Paint)

6.2.2 Procedure

6.2.2.1 Prepare the culture suspension from the standard culture as per the SOP for preparation of microbiological culture.

6.2.2.2 Select the dilution which will yield 10^4 to 10^5 cells per ml.

6.2.2.3 Take plate of different surfaces such as SS, Epoxy, panel, PU paint wall present in the clean room having a surface area of 25 cm^2.

6.2.2.4 From the previously determine suspension having 10^4 to 10^5 cells per ml inoculate one culture on different surfaces mention above.

6.2.2.5 With the help of sterile spatula spread the culture on surface.

6.2.2.6 Keep it on LAF bench for drying.

6.2.2.7 After the exposed duration and complete drying

(a) Spray the disinfectant

(b) disinfect the surface by wipe method.

6.2.2.8 Give a contact time of 0 min, 5min, 10 min, 15min, 20 mins and 30 mins.

6.2.2.9 The exact procedure for sanitization followed in clean room should be follow

6.2.2.10. With the help of a sterile moistened swab, swab the surface gently covering all the area of the surface.

6.2.2.11 Place the swab-stick in the test tube having a sterile saline solution and do not hold the swab more than 24hrs.

6.2.2.12 Vortex the test tube gently for 5 min.

6.2.2.13 Aseptically filter the sample through the 0.45 m meter membrane.

6.2.2.14 Give three washing of 100 ml each with 0.1% sterile peptone water /sterile water.

6.2.2.15 After the filtration with the help of sterile forceps take the membrane and place it on soybean casein digest agar.

6.2.2.16 Incubate the bacterial culture at 30 to 35 ^{0} c for 24 to 48 hrs. and fungal culture at 20 to 25^{0}c for 72 to 120 hrs.

6.2.2.17 After incubation count the number of colonies present on the membrane.

6.2.2.18 Proceed in the same manner taking all the culture to be tested with the mention

disinfectant.

7.0 Acceptance Criteria

7.1 The decrease in the bacterial load when exposed to the disinfectant shows that, the disinfectant is capable of decreasing the contaminate when used in the area that shall be minimum of 7-log reduction for non-spore forming microorganisms, yeast, molds with the decided the concentration.

7.2 Determine the contact period were the above said population log reduction of microorganisms achieved.

8.0 Observation

8.1 Record the observation of log reduction method in Annexure-I

8.2 Record the observation of surface spray, swab or wipe method in annexure-II

ANNEXURE-I

Name of the disinfectant							
Log reduction observed with contact period (in min)							
Name of organism	**0**	**5**	**10**	**15**	**20**	**30**	**Significant reduction observed**
Bacillus Subtilis(6633)							
Escherichia coli(4157)							
Staphylococcus aureus(6538P)							
P. aeruginosa(15442							
S. abony(6017)							
Candida albicans(10231							
Aspergillus brasiliensis (16404)							
Candida albicans(10231)							

ANNEXURE-II

❖ **Certificate Of Analysis**

Name of Product : ACITAR	
Lot No. / Batch No. : ATR 17014	Lot Size / Batch Size : 1500 Litres
Pack Size: (Cat. No.) : 5000 ml (ATR5000)	
Mfg.: Mar – 18	Exp. : Feb – 21
Date of Testing : 6th March 2018	Date of Release : 13th March 2018

Sr. No.	**Test**	**Specifications**	**Observations**	**Results**
1	Appearance	Clear Liquid	Clear Liquid	Complies
2	Physical State	Liquid	Liquid	Complies
3	Colour	Colourless	Colourless	Complies
4	pH	4.0 – 5.0	4.65	Complies
5	Odour	Characteristic Aldehyde	Characteristic Aldehyde	Complies

6	Water Miscibility	Miscible (Clear Solution)	Miscible (Clear Solution)	Complies
7	Specific Gravity at 27°C ± 2° C	1.008 – 1.018	1.014	Complies
8	Assay of Glutaraldehyde BP	(1.7 – 2.3) % w/v	2.1117 % w/v	Complies
9	Assay of Benzalkonium Chloride Solution IP	(4.5 – 5.5) % w/v	5.1259 % w/v	Complies
10	Microbiological Testing (viability Test)	ACITAR 5% v/v solution kills the following organism in 10 mins. *E. coil (ATCC 4157 /NCIM 2067)* *S. aureus (ATCC 6538P /NCIM 2079)* *P. aeruginosa (ATCC 15442 /NCIM 2862)* *B. Subtilis (ATCC 6633 /NCIM 2063)* *C. albicans (ATCC 10231 /NCIM 3471)* *A. brasiliensis (ATCC 16404 /NCIM 1196)*	ACITAR 5% v/v solution kills the following organism in 10 mins. *E. coil (ATCC 4157 /NCIM 2067)* *S. aureus (ATCC 6538P /NCIM 2079)* *P. aeruginosa (ATCC 15442 /NCIM 2862)* *B. Subtilis (ATCC 6633 /NCIM 2063)* *C. albicans (ATCC 10231 /NCIM 3471)* *A. brasiliensis (ATCC 16404 /NCIM 1196)*	Complies

- **CONCLUSION**

Firstly the viable count was calculated to determine the log capacity of the standard bacterial culture maintained in the organisation. After detection of the viable count the disinfectant ACITAR at a concentration 5% v/v showed more than 7 log reduction when tested via membrane filtration and direct inoculation method. After the conclusive evidence of 7 log reduction swab analysis was performed and the disinfectant ACITAR of concentration 5% v/v was found to be fruitful against the standard bacteria on different surfaces and later the activity of the disinfectant was monitored by agar diffusion method that proved if the concentration of disinfectant increases the zone of inhibition also increases. Hence it is concluded that after performing several test the results showed that the disinfectant was highly effective and could be used to different purposes at the sterile manufacturing facility during spillage and other hazardous situations.

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