ejpmr, 2018,5(4), 491-495

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

DEVELOPMENT AND VALIDATION OF MICROBIAL LIMIT TEST FOR FOSCARNET SODIUM API.

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Article Received on 14/02/2018 **Article Revised on 06/03/2018** **Article Accepted on 26/03/2018**

ABSTRACT

The Foscarnet Sodium is an important drug used to treat viral infections. Majorly it acts on (CMV) Cytomegalovirus and Herpes Simplex Virus I and II. Foscarnet Sodium has antimicrobial properties. It must undergo neutralization of antimicrobial compounds to allow microbial count testing according to recommendations by the official pharmacopoeias. The validation of antimicrobial activity neutralization and of the method for the microbial counting was performed according to USP 40. Lecithin soya used as neutralizer, Recovery levels over 70% of the microorganisms used in the test indicated the neutralization of antimicrobial activity and proved the absence of toxicity of neutralizer. The microbial counting method validated and proved accurately.

KEYWORDS: Microbial limit test, Foscarnet sodium, validation and development, soya lecithin.

INTRODUCTION

The microbial limitation testing is used to determine whether a product complies with an established specification for microbial quality. The test is design to estimate the number of viable aerobic microorganisms present in a product and define designated microbial species for products from the materials stage to final manufacturing product.

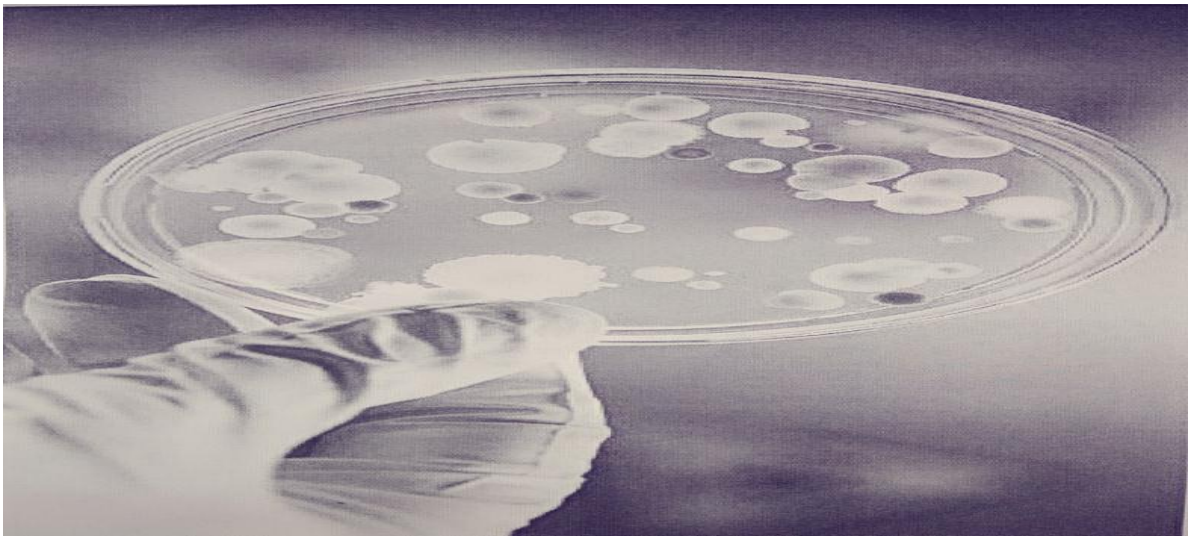

Figure a. Microbial Enumeration.

Scientific and technical advices in recent decades have enabled better development and control of drugs. Universal determine the minimum efficacy safety and quality requirements for medicines. (Carvalhoctal 2004, Bomblics and Weiss, Backmann 2007) drug quality is related to degree of compliance with such a requirements and minimum standards determine by the official standards.

To ensure these requirements are met it in necessary to physical, chemical and biological tests (Itah, Udokpoh, and Ofum 2004). Microbiological method performed for Foscarnet sodium by reference of USP 40. Method of

microbial counting seeks to assess the total number of bacteria, yeast and mold using specific culture media. This method includes a series of validations such as neutralization of antimicrobial agents, recovery of test microorganisms and the microbial counting method itself.

USP 40 and PDA technical report describe which key precision, accuracy, robustness and linearity parameters are to be observing in this validation. In addition another important factor is that the method implemented is validated for each product analyzed (USP 40 and Ramos 2010).

In this study Foscarnet sodium was investigated as one of the most widely used antiviral drug. Foscarnet sodium showing self-preservative activity. The conducting of validation test is for antimicrobial agent neutralization and for microbial counting method. These became key factor that will be prove the method effectiveness in recovering product contamination.

MATERIALS AND METHODS

To validate microbial counting method we used following media: Soya lecithin (Himedia Lot No. 06-1510). Soyabean casein digestive agar (Himedia Lot No. 294305). Soyabean casein digestive media (Himedia Lot No. 300950). Sabouraud dextrose agar (Himedia Lot No. 254260). Enterobacteria Enrichment Broth (Himedia Lot No. 172851). Rappaport Vassiliadis Salmonella Enrichment Broth (Himedia Lot No. 219510). MacConkey Broth (Himedia Lot No. 306735). Sabouraud dextrose broth (Himedia Lot No. 244647). Reinforced medium for clostridia (Himedia Lot No. 235379). MacConkey agar (Himedia Lot No.280892). Cetrimide Agar (Himedia Lot No. 229609). Manittol salt agar (Himedia Lot No. 186390). Violet Red Bile Glucose Agar (Himedia Lot No. 258010). Xylose Lysine Deoxycholate Agar (Himedia Lot No. 246276). Columbia Agar (Himedia Lot No. 250360).

Test Organisms

Table No. 1

Sr. No.	Name of Organism	ACTCC number
1	*Bacillus subtillis*	6633
2	*Escherichia coli*	8739
3	*Pseudomonas aeruginosa*	9027
4	*Staphylococcus aureus*	6538
5	*Salmonella abony*	NCTC 6017
6	*Clostridium sporogenes*	11437
7	*Candida albicans*	10231
8	*Aspergillus brasiliensis*	16404

Inoculum Standardization

From stock culture in Soyabean casein digest agar, bacterial cultures were transfer in to 10 ml of Soyabean casein digest medium, these cultures were incubated for 18 to 24 hrs. At 30˚C to 35˚C. Then centrifuge all inoculums, gave three 0.9% saline washings to obtain pure culture suspensions. Then serial dilutions are prepared in 0.9% saline solutions and the last four dilutions were plated and incubated at 30˚C to 35˚C. Then we prepared 10 – 100 cfu/ml suspension. Dilutions for yeast culturing are similar methodology to bacteria. In case of mold, the aspergillus brasilinsis was facilitated by suspending spores in 100 ml of 0.9% saline solution with supplemented with 0.05% of polysorbate 80. Serial dilutions plates of these cultures were incubated at 20˚C to 25˚C for 5 – 7 days.

Validation of Preservative Neutralization

Based on the drug compound, neutralization using 0.5% soya lecithin was chosen as recommended by the official compendia (USP 2017) and 1:10 dilution used for the water soluble products.

Determination of the efficacy and toxicity of the neutralizer is required to ensure the validation process of antimicrobial agent neutralization (Polyana Araujo de Assis 2011). This is observed through microorganism recovery in different groups of analysis, test, and peptone and viability group. Similar recovery between the test and peptone group demonstrate adequate neutralizer efficacy, similar recovery between the peptone and viability group demonstrate adequate neutralizer toxicity. This test was performed in duplicate and the results were used to demonstrate neutralization validation. The recovery of test microorganism to be greater than or equal to 70%.

Preparation of Analysis Group

The test (T) and peptone (P) are composed of 100 ml Soyabean casein digest broth supplemented with 0.05% soya lecithin. We added 10 gm of product sample in the test and adjust the pH between 6 – 8. The viability group contain only Soyabean casein digest broth from the analysis group we take 1 ml of aliquots were deposited in sterile petri plates simultaneously 0.1 ml of standardize suspension of test microorganism. The total aerobic microbial count (TAMC) and total yeast and mold count (TYMC) are determined using the validated pour plate method. While testing by using the pour plate method duplicate the SCDA plates for TAMC and duplicate the plates of Sabouraud Dextrose Agar (SDA) for TYMC were prepare. Petri plates keep on flat surface until the

plates solidify. Soyabean casein digest agar (SCDA) plates were incubated at 30˚C to 35˚C for 3 – 5 days, SDA plates incubated at 20˚C to 25˚C for 5 – 7 days. The recording the result was performing after completion period by counting the colonies using a colony counter and results express as cfu/gm.

Development and Validation of Microbial Counting Method

Microbial limit test was validated was validated to the parameters of precision, linearity, accuracy and robustness. The validation study is concluded in three independent experiments as described in USP<61>, the volume of inoculums added to each test tube should be within 1% of the volume of diluted product. The sample dilution was prepared as per individual development for microbial examination. We took 10 gm of Foscarnet sodium and dissolve in 100 ml of soybean casein digest medium supplemented with 0.5% of soya lecithin for total bacterial and fungal count for TAMC the SCDA plates were incubated at 30˚C to 35˚C for not more than 3 days, while SDA plates incubated at 20˚C to 25˚C for not more than 5 days.

Validation for Specified Microorganism

We had prepared sample dilutions as per individual development for microbial examination. We had 10 gm of Foscarnet sodium salt in 100 ml of Soyabean casein digest broth supplemented with 0.5% of soya lecithin (Stock solution). 10 ml of solution from above sample dilution forwarded to 100 ml of Enterobacteria Enrichment Broth (EEB) for the bile tolerant Gram Negative Bacteria. Each 10 ml of stock solution transfer to 90 ml of Soyabean casein digest medium (SCDM) separately for *E. coli, Pseudomonas Aeruginosa, Staphylococcus Aureus.* Transfer 10 ml of Foscarnet sodium Stock Solution to 100 ml of Sabouraud dextrose broth (SDB) for *Candila Albicans*. Transfer 10 ml of stock solution into two portions for the test of Clostridia. Separately use 10 gm of sample dissolve in 100 ml of Soyabean casein digest broth for test of *Salmonella*. These all samples are inoculated with test organisms as prescribed in USP 62 the growth was observed after 3 and 5 days for bacteria, 5 and 7 days for yeast and mold.

Table No. 2

Sr. No.	Name of Organism	ATCC	NCIMB	CIP	NBRC	NCTC
1	*Escherichi coli*	8339	8545	53.126	3972	NA
2	*Pseudomonas aeruginosa*	9027	8626	82.118	13275	NA
3	*Staphylococcus aureus*	6538	9518	4.83	13276	NA
4	*Salmonella abony*	14028	NA	80.39	NA	NA
5	*Clostridium sporogenes*	11437	12343	100651	NA	NA
6	*Candida albicans*	10231	NA	NA	1594	NA

Selective media and their usage

Table No. 3

Sr. No.	Name of Test Microorganisms	Medium	Incubation Temperature	Incubation Time
1	*Bile Tolerant Gram Negative Bacteria*	Enterobacteria Enrichment Broth (EEB) Voilet Red Bile Glucose Agar (VRBGA)	30˚C to 35˚C. 30˚C to 35˚C	24 to 48 hrs 18 to 24 hrs
2	*Escherichi coli*	MacConkey Broth (MB) MacConkey Agar (MA)	42˚C to 44˚C 30˚C to 35˚C	24 to 48 hrs 18 to 72 hrs
3	*Salmonella abony*	Rappaport Vassiliadis Salmonella Enrichment Broth (RVSEB) Xylose Lysine Deoxycholate Agar (XLDA)	30˚C to 35˚C 30˚C to 35˚C	18 to 24 hrs 18 to 48 hrs
4	*Pseudomonas aeruginosa*	Cetrimide Agar (CA)	30˚C to 35˚C	18 to 72 hrs
5	*Staphylococcus aureus*	Manittol Salt Agar (MSA)	30˚C to 35˚C	18 to 72 hrs
6	*Clostridia*	Reinforced medium for Clostridia (RCM) Columbia Agar (COA)	30˚C to 35˚C 30˚C to 35˚C	48 hrs 48 to 72 hrs
7	*Candida albicans*	Sabouraud Dextrose Broth (SDB) Sabouraud Dextrose Agar (SDA)	30˚C to 35˚C 30˚C to 35˚C	3 to 5 days 24 to 48 hrs

RESULT AND DISCUSSION

The Neutralization effectiveness was observed by the high recovery percentage in the test group which is ranged from 90% to 99% for Foscarnet Sodium. Along with recovery determination the toxicity of neutralizer was confirm by the recovery rates of microorganism in the peptone group which ranged from 91% to 115%. During validation time we find antimicrobial activity due

to which growth of microorganisms were inhibited, we tried different dilutions of neutralizing agent on different concentrations, finally we got more than 70% recovery while testing with 0.5% soya lecithin. We had done toxicity analysis for 0.5% soya lecithin with the challenging microorganisms prescribed in USP 61. Here also we got satisfactory results. The efficacy and toxicity of neutralizer was confirmed by recovery rates.

Neutralization Evalution

Table No. 4

Sr. No.	Name of Microorganism Used	Analysis Group		
		V (x)	T (x)	% R
1	*Bacillus Subtilis*	25	24	96
2	*Pseudomonas aeruginosa*	16	14.5	90.6
3	*Staphylococcus aureus*	34.5	35	101.4
4	*Candida albicans*	37.5	34	90.6
5	*Aspergillus brasiliensis*	40.5	38.5	95

Note: Viability (V), Test (T), Recovery Percentage (%R)

Toxicity Evaluation

Table No. 5

Sr. No.	Name of Microorganism Used	Analysis Group		
		V (x)	P (x)	% R
1	*Bacillus Subtilis*	25	30.5	115
2	*Pseudomonas aeruginosa*	16	15.5	96.8
3	*Staphylococcus aureus*	34.5	32	92.7
4	*Candida albicans*	37.5	38.5	102
5	*Aspergillus brasiliensis*	40.5	38.5	95

Note: Viability (V), Peptone (P), Recovery Percentage (%R).

Validation of microbial counting method

The validation study must show that recovery of the inoculums content less than 100 cfu of the representative organism is not inhibited by the test sample. The microbial counting method provide accurate on both parameters the accuracy is defined by USP as the degree of data approximation obtained in the analysis. Precession is usually expressed by relative standard deviation or coefficient of variation. The test solution was inoculated with *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Candida albicans, Aspergillus brasiliensis* and incubated as specified in the individual development for microbiological examination. It should be observed that the incubation time for product testing is two days longer than the conditions used in the validation of method. This longer incubation time provides a better survival condition for damage or slow growing cells. The growth observed 3 and 5 days for bacterial, 5 and 7 days for yeast and mold the product is not inhibiting to the challenged organism hence product is likely to be get contaminated with above said microorganisms. The sample Foscarnet sodium was enumerated by using pour plate method. The average pour plate counts are determined for the replicate plates and results are reported as the number of cfu per gram. Dilution factor is applicable to result. We diluted product 1:10 times so we gave result by applying dilution factor 10.

Validation for specified microorganisms

The sample Foscarnet Sodium was tested for specified microorganisms based on USP 62. The specified microorganisms such as *Escherichia coli, Salmonella abony, Pseudomonas aeruginosa, staphylococcus aureus, Clostridia and albicans.* The specified microorganisms were recovered during the testing indicated as a positive for the tests specified as per the acceptance criteria. Hence is likely to get contaminated with the above mentioned microorganisms.

Table No. 6

Sr. No.	Name of the Specified Microorganisms	Results of Specified Microorganisms
1	Bile tollerent Gram Negative Bacteria	Growth observed
2	*Escherichia coli,* ATCC8739	Growth observed
3	*Salmonella abony,* NCTC6017	Growth observed
4	*Pseudomonas aeruginosa,* ATCC9027	Growth observed
5	*Staphylococcus aureus,* ATCC 19404	Growth observed
6	*Candida albicans,* ATCC 10231	Growth observed

CONCLUSION

The method validation process was established for document evidence which provided a high degree of assurance. That the specified method will consistently provide accurate results. Based on the studies carried out it is concluded that there is a antimicrobial activity was neutralized with 0.5% of soya lecithin in the product and test organisms prescribed in USP 61 and 62 are recovered during the testing indicating as a positive results for the tests specified as per the acceptance criteria. While testing the Foscarnet Sodium, need to adjust the pH in between 6.0 to 8.0. The aim of study was to validate the microbial limit test results which can be interacted satisfactorily given the wide variability of microorganism involved in method validation.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Emcure Pharma. Ltd. for the samples of pure Foscarnet Sodium for supplying gift samples and also thankful to Principal D. N. R. College of PG and Research Center, Bhimavaram, Andhra-Pradesh. For the providing all the facilities to carry out the research work successfully.

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