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VALIDATION OF MICROBIOLOGICAL CONTROL METHOD OF DIAZEPAM - RECTAL SOLUTION 5 MG/2.5ML

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ABSTRACT
Bioburden testing is a quality control process used during production to quantify microbial contamination in finished products to ensure the safety of a manufactured product. This study represents validation of bioburden testing of Diazepam rectal solution according to the requirements of European Pharmacopoeia. The aim of this study was to validate the method for bioburden testing capable of detecting any microbiological contamination by spread-plate method of finished product Diazepam 5 mg/2.5 ml – rectal solution which is a pharmaceutical drug product produced in Profarma Sh.a. The plates of casein soya bean digest agar at 30-35 °C for 3 days and the plates of Sabouraud-dextrose agar at 20-25 °C for 5 days were incubated. For the spread-plate method, the manual reading as a method to perform the colony enumeration was used. The recovery rate and a mean count of any of the test organisms were calculated. Growth obtained was acceptable, thus the media tested were considered suitable for use at the method evaluation. The mean count of any of the test organisms was not differing by a factor greater than 2 from the value of the control. The growth of the positive control was visually comparable to the one with sample spiked. Hence, the product passed the bioburden study and did not have inhibitory effect on the microbes/contaminants. The spread-plate method was found to be precise and accurate for enumeration of micro-organisms in rectal solution Diazepam.

KEYWORDS: diazepam, microbiological contamination, bioburden testing, spread-plate method, enumeration.

INTRODUCTION
Bioburden testing is a quality control process used during production to quantify microbial contamination in water, raw materials, or finished products to ensure the safety of a manufactured product. For non-sterile products, European guidelines require a certain bioburden to be met at the point that a final product passes the test. This article represents validation of bioburden testing (spread-plate method) of Diazepam rectal solution according to the requirements of European Pharmacopoeia.[1-5]

AIM
To validate the method for bioburden testing capable of detecting any microbiological contamination by spread-plate method of finished product Diazepam 5 mg/2.5 ml – rectal solution which is a pharmaceutical drug product produced in Profarma Sh.a.

MATERIALS AND METHODS
Instruments and materials
Diazepam samples were obtained by Profarma company. To prepare samples, the following equipments were used: analytical balance (Explorer OHAUS, NY, USA); Petri plates glass (Isolab, Laborgeräte, Germany); a vortex mixer IKA MS 3 (IKA-Werke, Germany) and semi-Automatic Laboratory Sterilizer model 3850 MLV (Tuttnauer, Breda, The Netherlands). The study was carried out using a microbiology safety cabinet (Euroclone Top safe, Lombardia, Italy) and incubators (Memmert, Germany). Set of lyophilized cultures (containing 10-100 CFU/ml EZ-Accu Shot); Set Lot 8172-130; Ref. 8172 [*S. aureus* (Lot 485-1048-1); *P. aeuruginosa* (Lot 484-1402-4); *Bacillus subtilis* (Lot 486-1261-3); *Candida albicans* (Lot 443-1289-4); *Asperigillus brasiliensis* (Lot 392-1234-2)] were supplied by Microbiologist. Trypticasein Soy Agar EP/USP/ISO and Saboraud Dextrose Agar 4% were supplied by Condalab. All the materials used in the study had absence of toxicity for micro-organisms. Also they are compatible with the product.

Microbiological procedure
The bioburden testing was carried out under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions were taken

to avoid contamination that they do not affect any micro-organisms that are to be revealed in the test. The product to be examined has no antimicrobial activity.

In choosing samples of product for determination of bioburden the final product was taken randomly. A sample size of 10 items for monitoring of bioburden levels was used. The study product is classified as a water-soluble product. Diazepam final product has alcohols (ethanol 96%, propylene glycol, benzyl alcohol) as interfering substances and it is recommended to use dilution as a potential neutralizing method. After developing a method, a suitable neutralizing agent was found. The sample was prepared by dissolving 10 mL of product under test in 100 mL of buffer sodium peptone water pH 7.0. Further dilutions (1:100 and 1:1000) were prepared with the same diluent. Aseptically was transferred 1 mL of the pretreated specimen in duplicate in sterile Petri dishes. For reproducibility of the results the same exercise was worked out thrice. For each medium at least 2 Petri dishes for each level of dilution were prepared. The plates of tryptic soya agar (TSA) at 30-35 °C for 3 days and the plates of Sabouraud-dextrose agar (SDA) at 20-25 °C for 5 days were incubated. The control compendial media were used with manual analyst readings and incubations. For the spread-plate method, the manual reading as a method to perform the colony enumeration was used. For positive product controls, each of culture suspension (0.1 mL that contain 10-100 CFU) was added to the sample. To each plate, the respective media were added at a temperature not more than 50 degree Celsius. After the agar was hardened, aseptically was pipetted out 1 mL of the spiked sample in duplicate Petri plates. The Petri dishes were gently swirled to have a proper mixture of the solution throughout the agar. The plates were inverted and incubated (TSA plates at 30-35 degree Celsius for 3 days and SDA plates at 20-25 degree Celsius for 5 days). The number of micro-organisms recovered from the prepared sample diluted and incubated following the procedure, was compared to the number of micro-organisms recovered from the control preparation.

The plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC (Total Aerobic Microbial Count) and 50 for TYMC (Total Yeast and Mold Count) were selected. The arithmetic mean per culture medium of the counts and the number of CFU per milliliter of product were calculated. To minimize any variability in the manual enumeration method, three analysts read the positive controls, negative controls, and test samples after the incubation. The recovery rate and a mean count of any of the test organisms were calculated.

RESULTS

No growth of micro-organisms were present in negative control. Growth obtained was acceptable, thus the media tested were considered suitable for use at the method evaluation of the microbiological control. The media selected for the bioburden test did not have any inhibitory effect on the microbes/contaminants. The mean count of any of the test organisms was not differing by a factor greater than 2 from the value of the control. The recovery rate and a mean count of any of the test organisms are presented in Table 1.

Table 1. Recovery of challenge microorganisms in the presence of sample.

			Results CFU*		Recovery (%)			
Test Sample	**Dilution of the Sample**	**Incubation Temperature**	**Lot 20 07**	75%	**Lot 21 05**	87%	**Lot 20 04**	93%
PPC (product+ *S.aureus*)	1:10	30-35	30		35		28	
PC (blank+ *S.aureus*)			40		40		30	
PPC (product+ *B.subtilis*)		30-35	30	75%	29	96%	29	90%
PC (blank+ *B.subtilis*)			40		30		32	
PPC (product+ *P.aeruginosa*)		30-35	49	98%	45	90%	47	94%
PC (blank+*P.aeruginosa*)			50		50		50	
PPC (product+ *C.albicans*)		30-35	20	66%	30	76%	30	75%
PC (blank+ *C.albicans*)			30		39		40	
PPC (product+*A.braziliensis*)		30-35	46	97%	45	90%	40	80%
PC (blank+ *A.braziliensis*)			47		50		50	
PPC (product+ *C.albicans*)		20-25	40	80%	40	81%	29	74%
PC (blank+ *C.albicans*)			50		49		39	
PPC (product+ *A.braziliensis*)		20-25	39	78%	35	73%	50	100%
PC (blank+ *A.braziliensis*)			50		48		50	

*Average value; PPC
Positive Product Control
PC- Positive Control

DISCUSSION

No growth of micro-organisms was present in negative control. Growth obtained was acceptable, thus the media tested were considered suitable for use at the method evaluation of the microbiological control. The media selected for the bioburden test did not have any inhibitory effect on the microbes/contaminants. The total aerobic microbial count (TAMC) in the product was less than 200 cfu/mL. The total combined yeasts/mold count (TYMC) in product was less than 100 cfu/mL. The comparison between the number of micro-organisms recovered from the prepared sample and the number of the micro-organisms from the control preparation, did not show any inhibition. After verifying the suitability of the spread-plate method, the mean count for all the test organisms did not interfere by a factor greater than 2 from the value of the control in the absence of product. The recovery rate has met the acceptance criteria.

CONCLUSIONS

The peptone sodium buffer pH 7.0 used as diluent for the bioburden testing did not have inhibitory effect on the microbes/contaminants. The growth of the positive control was visually comparable to the one with sample spiked. Hence, the product passed the bioburden study and did not have inhibitory effect on the microbes/contaminants. The spread-plate method was found to be precise and accurate for enumeration of micro-organisms in rectal solution Diazepam.

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CONFLICT OF INTEREST

The author declares no conflict of interest in preparing this article.

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