Tampereen ammattikorkeakoulu

# Validation of a Method for Analysing Rinsing Waters from Production of Ophthalmic Solutions

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VIINAMÄKI, JENINA:
Huuhteluvesien analysointiin käytetyn menetelmän validointi silmälääkkeiden tuotannossa

Opinnäytetyö 33 sivua, joista liitteitä 6 sivua.
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Opinnäytetyö tehtiin alkuvuodesta 2022 NextPharma Oy:n Tampereen toimipisteen mikrobiologiseen laadunvalvontalaboratorioon. Tämä tutkimus sisältää luottamuksellista tietoa, jotka on jätetty pois työn julkisesta versiosta. Työn tavoitteena oli validoida yrityksessä käytetty menetelmä koskien tuotannon huuhteluvesien mikrobiologista analysointia. Huuhteluvedet analysoitiin menetelmällä, joka pohjautui Euroopan farmakopean lukuun 2.6.12 ja Yhdysvaltojen farmakopean lukuun 61. Tavoitteena oli varmentua siitä, että menetelmä itsessään ei ole antimikrobinen, vaan mahdolliset tuotannon huuhteluvesien mikrobit ovat havaittavissa menetelmää käyttäen.

Opinnäytetyön tarkoituksena oli luoda näyte vastaamaan oikeita tuotannon huuhteluvesiä lisäämällä siihen suurin sallittu määrä lääkeainetta ja tuotannossa käytettävää pesuainetta sekä säilöntäainetta. Näyte altistettiin eri mikrobeille, jotka ovat yleisiä kontaminaation aiheuttajia lääketeollisuudessa. Mikrobisuspensiot valmistettiin laimentamalla American Type Culture Collection -mikrobikannoista. Näyte suodatettiin kalvosuodatusmenetelmällä ja sen mikrobikasvustoa analysoitiin. Analysoitava validointiparametri työssä oli mikrobiologinen saanto.

Työlle asetetut tavoitteet saavutettiin. Menetelmä ei osoittanut validointiprosessin aikana antimikrobisia ominaisuuksia käytetyllä näytteellä, vaan mikrobien saannot olivat pääosin sallituissa rajoissa. Sallittujen rajojen ulkopuolella olevat kaksi tulosta koettiin johtuvan inhimillisistä virheistä, kuten virheellisestä mikrobisuspensioiden valmistuksesta, pipetoinnista tai pesäkemäärien lasku- ja havaitsemisvirheistä.

Jatkossa menetelmässä käytetään erilaista suodatinkalvoa kuin ennen, jotta menetelmä olisi luotettavampi. Menetelmää voitaisiin tulevaisuudessa validoida käyttäen myös muilla validointiparametrejä, kuten häiriöalttiutta. Näin menetelmästä saataisiin entistä luotettavampi.

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Asiasanat: validointi, kalvosuodatusmenetelmä, antimikrobiset yhdisteet

**ABSTRACT**

Tampereen ammattikorkeakoulu
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VIINAMÄKI, JENINA:
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This Bachelor's thesis was made at Microbiological Quality Control laboratory of NextPharma Oy –Tampere site. This study contains confidential information that is excluded from the published version. The purpose of this study was to validate a microbiological method used to analyse production rinsing waters. The method to be validated was based on European Pharmacopeia's chapter 2.6.12. and United States pharmacopeia's chapter 61. The aim was to state that the method itself is not antimicrobial but the possible contaminants in the rinsing waters from production are easily discernible.

The objective of this study was to create a sample that is equivalent to the real production rinsing waters by adding the maximum allowable amount of the active pharmaceutical ingredient, cleaning agent and preservative to the sample. The sample was then challenged with different microbes that are common contaminants in the pharmaceutical industry. The challenge microbes were prepared by dilution from American Type Culture Collection strains. The sample was filtered with a membrane filtration method and the microbial growth found in it was analysed. The validation parameter analysed in this study was microbial recovery.

The goals set for this study were achieved. The method did not show antimicrobial properties during the validation process and microbial recoveries were mainly within the limits of acceptance. Results that did not meet the acceptance criteria were perceived to be the result of a mistake made in the preparation of challenge organisms, pipetting or from the calculation or the detection of colonies.

From now on, a different membrane is used in the filtration process for more reliable analysis. The method could be analysed also with different validation parameters to make the method more dependable. Validation parameters examined in future could be for example robustness.

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Key words: validation, membrane filtration method, antimicrobial substances

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## ABBREVIATIONS

| | |
|---|---|
| API | Active Pharmaceutical Ingredient |
| ATCC | American Type Culture Collection |
| BAC | Benzalkonium chloride |
| CFU | Colony Forming Unit |
| DNA | Deoxyribonucleic acid |
| GMP | Good Manufacturing Practice |
| Ph. Eur. | European Pharmacopoeia |
| PVDF | Polyvinylidene fluoride |
| QA | Quality Assurance |
| QC | Quality Control |
| SDA | Sabouraud Dextrose Agar |
| TSA | Trypticase Soy Agar |
| TSB | Trypticase Soy Broth |
| USP | United States Pharmacopoeia |
| WFI | Water for Injection |

# 1 INTRODUCTION

This Bachelor's thesis was conducted in Microbiological Quality Control (QC) laboratory of NextPharma Oy – Tampere site. In Tampere, NextPharma is a contract manufacturer of pharmaceuticals focusing on ophthalmic solutions. They also provide pharmaceutical development and analytical services to customers all around the world.

Ophthalmic solutions in NextPharma are being manufactured in compounding tanks and filling needles. The tanks and needles are washed with specific water and cleaning agent to prevent hazardous micro-organisms from growing inside the instruments. Due to the usage of these instruments, tiny amounts of Active Pharmaceutical Ingredient (API) and preservative are left inside the tank and filling needle, before final washing. Because of that, the rinsing water not only consists of purified water and cleaning agent, but also API and preservative.

The objective of this validation project is to state that the rinsing water used to wash compounding tanks and filling needles in the production of ophthalmic solutions has no antimicrobial effect when being tested according to European Pharmacopoeia (Ph. Eur.) chapter 2.6.12 and United States Pharmacopoeia (USP) chapter 61 membrane filtration method. This validation verifies the efficiency of NextPharma Oy's method for analysing production rinsing waters. Method to be validated for this study is based on Ph. Eur 2.6.12 and USP 61.

The purpose of this project is to challenge the sample like an actual rinsing water to different microbes and observing the growth of them. This is conducted by filtrating the sample through a membrane and rinsing the membrane with a solution containing culture medium and the challenge microbes. Some information that is included in this study has been omitted from the published report due to confidentiality.

# 2 THEORETICAL BACKGROUND

## 2.1 Pharmaceutical regulations

All work regarding the production of pharmaceuticals needs to be founded on Good Manufacturing Practice (GMP). GMP narrates the standards used in pharmaceutical industry and sets up requirements for the final product to ensure its safety, quality and appropriateness. Every pharmaceutical manufacturer aiming at or working in the European Union market, needs to observe GMP requirements. (European Medicines Agency n.d.) Having strict regulations concerning the manufacturing process of pharmaceuticals ensures the safety of medicines and medical devices to the consumer and to the environment.

According to the GMP regulations, to work with sterile medical products, the personnel must have adequate education and qualifications that supplies the people work environment. Employees need to be familiarized properly for the safe work in various parts of the production by the production facilities of the company. If the employee needs to work in cleanrooms, where the medical products are being prepared, the employee is trained to work aseptically to minimize the risks of contamination. Only proficient personnel can enter the pharmaceutical production area. The qualifications are kept up to date with education organized at regular intervals. (EU Commission 2020.)

All equipment, methods and facility need to be validated, qualified and optimized to correspond the GMP guide. (EU Commission. 2020.) The maintenance of production's qualification is monitored by taking samples regularly from different surfaces, personnel and the air inside the facility. Every raw material and production instrument coming from outside the production site is being tested in the microbiological QC laboratory for possible extrinsic contaminants. All samples are being taken and analysed by educated personnel.

All documentation regarding any activity in the manufacturing of pharmaceuticals should be clear, contain no errors and be kept up to date. Every procedure needs to be documented properly so that tracing the history of each batch of a medical

product is possible. All documents concerning a single batch of a medical product, need to be retained for at least one year after the batch's expiry date. (Directive 2003/94/EC.) If any problem in the medical product occurs, the possible source of the problem can be traced, and possible product recall can be organized.

This validation is based on the pharmacopoeias of the United States and European Union. A pharmacopoeia is a publication that contains the mandatory regulations for medicinal products and substances, adjuvants and excipients. (Fimea n.d.) Different countries and unions have their own pharmacopoeias which regulate the pharmaceutical industry in the particular country. Pharmacopoeias are updated regularly to assure the safety of pharmaceuticals produced to consumers and to the environment.

## 2.2 Microbiological method validation

Validation signifies that an analytical method fulfils the requirements set for it. The requirements can be set by the laboratory itself or the requirements can be based on law or other regulations. The method is tested using different validation parameters that represent the dependability of the method. Validation parameters can be for example repeatability, uncertainty and linearity. (Hägg 2016, 7). In this method validation, the validation parameter examined is microbial recovery.

Microbiological method validation delineates the possible antimicrobial properties of the sample or the method itself. When a substance is antimicrobial, it kills microbes. For example, cleaning agents, medical substances and preservatives are antimicrobial. Microbial validation also determines the capability of incubation and other microbial growth conditions to recover microbes. (Sandle 2015).

A validation protocol is formulated always before starting to work with the analysis. In outline of the protocol the objective, the sample, validation parameters, time schedule and the used instruments for the validation are described. The requirements for the validation to be accepted, are also mentioned and explained in the protocol. When planning the validation well, all the excess work regarding

the possible mistakes found in the method, can be minimized. (Hägg 2016, 9–14).

After the analysis, a validation report is conducted. In the report, all changes made in the analysis are presented and the results are shown. Based on the results, the method is commented and the possible improvements for the method are told. Also, the requirements for the validation are gone through and the future of the method is observed. (Hägg 2016, 15–16).

### 2.3 Microbial recovery

Recovery in validation denotes the amount of analyte added to the sample compared to the amount of the analyte after the analysis. Recovery is presented with percentual numbers. When recovery is 100%, no analyte is disappeared during the analysis. Recovery can also be over 100% for example when viable microbes are used as the analyte. This is called microbial recovery. The number of challenge microbe colonies on the sample plates, should be the about same than in the control plates. Microbial recovery thus denotes the amount of challenge microbes recovering from the analysis. Microbial recovery is calculated as per equation (1) (Hägg 2016, 29).

$$\text{Recovery} - \% = \left(\frac{\text{specified concentration}}{\text{added concentration}}\right) \times 100\%, \tag{1}$$

where specified concentration is the number of challenge microbe colonies on the control plate and the added concentration is the number of colonies on the sample plates. The division is multiplied with 100%, to get the percentual value of recovery.

## 2.4 Microbial membrane filtration method

The microbial membrane filtration method is used widely in pharmaceutical industry for its sensitivity and reliable analysis. It is usable for specimens that are soluble. This method is based on the ability of microbes to penetrate through the membrane into the filtrate. In the membrane filtration method, the risk for extrinsic contamination is low due to the minimum product interference during the analysis. (Clontz 1998, 66–69). The membrane filtration method is used in this study to determine the microbial growth of the filtrate samples.

In the membrane filtration method, the sample is filtered first through a certain selected membrane. The membrane is selected according to the analysed substance. To facilitate the filtration, a pump or water suction is used. The membrane is rinsed multiple times with a rinsing solution containing culture medium, usually peptone water. Rinsing the membrane with culture medium solution removes the possible antimicrobial substances from the surface of the membrane that could inhibit the growth of challenge microbes. The rinsed membrane is transposed to culture medium and incubated. (Clontz 1998, 66–69).

## 2.5 Levofloxacin

Active pharmaceutical ingredients (APIs) in medical products are the substances that produces the therapeutic effect of the medicine. For example, in antibiotics, the API is the substance that kills the bacteria that has caused the infection.

Levofloxacin is a widely used antibiotic, classified as third generation fluoroquinolone (Figure 1). It is the S-enantiomer of another widely used antibiotic, ofloxacin. (Johnson, D. S. & Li, J. J. 2007, 47.) Levofloxacin was approved by United States Food and Drug Administration in 1996 and after that, levofloxacin has reached enormous commercial success selling in 2004 for over 2.33 billion U.S. dollars. (Collin, F., Karkare, S. & Maxwell, A. 2011)

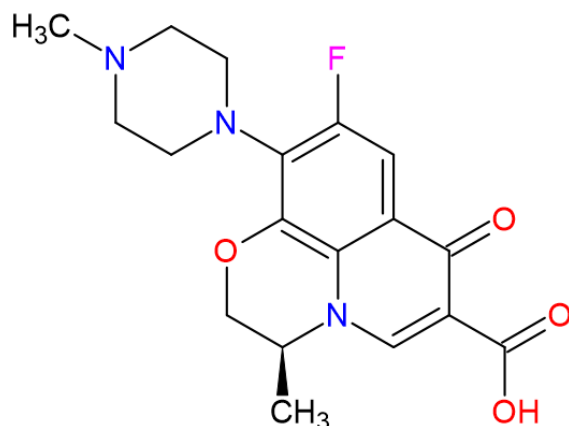



FIGURE 1. Chemical structure of levofloxacin.

Fluoroquinolone antibiotics such as levofloxacin, can be modified structurally to accomplish certain goals. Because of this, fluoroquinolone antibiotics act effectively against multiple aerobic Gram-negative and Gram-positive microbes. (Reese, Betts & Gumustop 2000, 544–545) They are used for respiratory infections, urinary tract infections and eye infections. Levofloxacin is mostly administered orally and ocularly in tablet form or in oral solution. Despite the wide use of levofloxacin, very little number of bacteria are resistant to this antibiotic. (Anderson & Perry 2008, 536–537)

The medicinal effect of levofloxacin and other fluoroquinolones is based on inhibiting the target bacteria's deoxyribonucleic acid (DNA) gyrase enzyme. (Reese, Betts & Gumustop 2000, 544–545) DNA gyrase enzyme is found in every cell type and is viable to the cell by uncoiling the DNA for replication. Fluoroquinolones work by forming complexes with DNA. Fluoroquinolone–DNA complex inhibits the possibility of replication in DNA and so new bacterial cells are not being formed and the bacterium dies. (Johnson & Li 2007, 43–44.)

## 2.6 Benzalkonium chloride

Benzalkonium chloride (BAC) is a disinfectant and antiseptic that is used in various applications all over the world for example in agriculture, cosmetics and in pharmaceutical industry. (Figure 2.) In pharmaceutical industry, BAC is used as preservative to increase the durability of certain medicines. It has antimicrobial properties to multiple microorganisms and so it is commonly used. (Merchel Piovesan Pereira & Tagkopoulos 2019.)

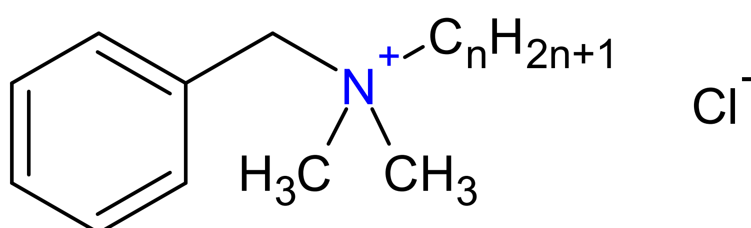



FIGURE 2. Chemical structure of benzalkonium chloride.

In 2014, European Union forbade the use of BAC in pesticides due to the feasible residues in food by this compound. (Regulation 1119/2014.) The safety of BAC to humans is controversial, however most studies state that the substance does not have detrimental effect on humans or environment, in small amounts. (Merchel Piovesan Pereira & Tagkopoulos, 2019.)

## 2.7 Deconex® CIP alpha-x

Deconex® is a brand from Swiss cleaning chemical manufacturer Borer Chemie AG. Deconex® CIP alpha-x is a powerful alkaline cleaner for pharmaceutical industry and other chemical industries such as cosmetics and biotechnology. (Borer Chemie AG. 2008.)

Deconex® CIP alpha-x is designed for validated cleaning, and it allows the validation to be as efficient and high-quality as possible. The cleaning agent is suitable for every GMP compliant surfaces in the pharmaceutical industry. (Borer Chemie AG. 2008.)

This cleaning agent's cleansing effect is based on the strong alkalinity of the chemical. Its pH is approximately 12.5 and due to this, it is used at diluted concentrations of 0.5–2.0 % (v/v). Occupational health and safety regulations are always acknowledged when working with Deconex® CIP alpha-x. (Borer Chemie AG. 2008.)

# 3 VALIDATION

## 3.1 Validation procedure

This validation is based on a method used in NextPharma Oy for analysing production rinsing waters. The validation was performed according to validation protocol. The protocol was written out and approved by Quality Assurance (QA) of NextPharma Oy before the analysis. After the validation, a validation report was conducted based on the execution of the validation and the results. QA also approved this document. The validation is required to be carried out by the GMP regulations and the regulations set by NextPharma Oy. Qualifications concerning the experimental part of the validation were procured.

In this method validation, sample solutions are being tested by the membrane filtration method with challenge microbes and different rinsing techniques. Every filtrate sample will have three collateral samples to achieve as authentic results as possible. Membranes are transferred onto Tryptic Soy Agar (TSA). TSA was chosen for this validation due to its decent amount of nutrients for as many microbes as possible to grow on.

All steps need to be performed using sterile equipment. To prevent extrinsic microbial contamination, a hairnet, sterile gloves and a separate work jacket are worn when working with the analysis. The expiration date of all equipment and substances is ensured before starting to work with the analysis. Water used primarily in this validation, was Water for Injection (WFI).

All microbial control plates must have the instructed number of colonies per plate. If not so, the suspensions are remade. Recovery of the challenge microbes is accepted when the recovery on the test plate is within 50–200 % of the control. There must not have microbial growth in negative control or product control plates.

## 3.2 Membrane Filtration

The membrane filtration method was chosen for this analysis because the sample was in liquid form and easily filtered. This method is also used in the process of analysing rinsing waters from the production of pharmaceuticals at NextPharma Oy. The membrane filtration method is presented as suitable microbial enumeration method in both USP 61 and Ph. Eur. 2.6.12. (European Pharmacopoeia 2.6.12. & United States Pharmacopoeia 61)

Validation was performed using Millipore Microfil® -filtration funnel. (Picture 1.) This filtration funnel used water suction to help with the filtration. Funnel and its rubber tube were disinfected carefully with 70% ethanol and the three filtration heads of the funnel were flamed before the analysis to prevent undesired extrinsic contamination.

PICTURE 1. Millipore Microfil® -filtration funnel.

When analysing real production rinsing waters in NextPharma Oy with the membrane filtration method, a cellulose ester membrane is used. This membrane is not the greatest to use for this analysis because the filter size of this membrane is small and the antimicrobial substances are at risk of sticking into the surface of this membrane and killing the possible microbes attached to it. Because of that,

a different membrane was used for this analysis. The membrane used was a polyvinylidene fluoride (PVDF) membrane that has greater filter size, 0.45 µm, which is enough for the antimicrobial substances to pass through. After the funnel's disinfection, sterile filtration disks were placed on the filtration heads. (Picture 2.) The PVDF membrane was placed on top of the filtration disks.

PICTURE 2. Filtration disks.

On top of the PVDF membrane, a plastic filtration cup, to where the sample and rinsing solutions were poured, was attached. The sample was filtrated first. According to the method used to analyse real rinsing waters, the sample amount needed to be rinsed, is 100 ml. When taken the 10% sample overage into consideration, the total amount of sample needed to rinse, is 110 ml. This volume was not exact when performing the analysis and the real amount of sample filtered can depart from the instructed.

After the filtration of the sample, the membranes were rinsed three times with 100 ml of 0.1% peptone water and collateral membranes five times with 100 ml of 0.1% peptone water 0.1% polysorbate 80. To the final rinsing solution, 100 µl of microbial suspension containing 10–100 cfu, was added. Membranes were then placed on TSA agar plates and incubated at 30–35 ºC for 5 days.

In between every sample, the filtration heads were disinfected with 70% ethanol and flamed. Also, the table surrounding the funnel and used pipettes were wiped thoroughly with 70% ethanol. Forceps were changed to new sterile ones and the contaminated ones were placed on a different table to prevent contamination for the next sample.

### 3.3 Sample

The sample for this validation project was made to represent the worst-case conditions. This means that real rinsing waters from compounding tanks and filling needles were not used because the levels of product residues in them are low or non-existent. Instead, using a solution containing a maximum allowable level of API, cleaning agent and preservative, the effectiveness of the method is more easily discernible. A sample of this kind was made manually for the analysis.

The sample contained 0.04 µg/ml of levofloxacin as API, 0.054 µg/ml of BAC and 6.7 µg/ml of Deconex® CIP alpha-x. Levofloxacin was chosen for this study due to its wide use in the pharmaceutical industry and its availability. Levofloxacin is one of the most used API in NextPharma Oy. BAC and Deconex® CIP alpha-x were chosen for this study because they are commonly used in NextPharma Oy.

Sample components were diluted in WFI. Levofloxacin dissociates easily due to the strong alkalinity of Deconex® CIP alpha-x. Because of that, two different sample solutions were made, one containing WFI and levofloxacin and the other one containing WFI, Deconex® CIP alpha-x and BAC. The two sample solutions were analysed separately. If combining all substances used in the real rinsing water of production instruments together, the sample solution would not be valid.

All the sample components were weighed out by a chemical QC laboratory of NextPharma Oy. Sample solutions were manufactured on the same day as the analysis to assure the validity of the method. All the solutions were prepared in autoclaved volumetric flasks. Three litres of each sample solution were made. Validation was performed with 10% sample overage which is part of the validation

procedure at NextPharma Oy. By using excess sample volume, possible inaccuracies during filtration and sample handling are being covered.

## 3.4 Preparation of challenge microbes and rinsing solutions

### 3.4.1 Preparation of challenge microbes

Challenge microbes are used in validation to show that the method itself does not have antimicrobial properties. All the challenge microbes used in this study were American Type Culture Collection (ATCC) strains. Microbes used for this analysis, were *Aspergillus brasiliensis*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Detailed information regarding these microbes is found in table 1. This group of microbes includes a mould and a yeast, Gram-negative and Gram-positive microorganisms for the analysis to be as comprehensive as possible. These organisms were chosen for this analysis based on Ph. Eur 2.6.12 and USP 61. (European Pharmacopoeia 2.6.12. & United States Pharmacopoeia 61)

TABLE 1. Challenge microbes.

| Microbe | Lot | Manufacturer | Number of passages | Expiration date |
|---|---|---|---|---|
| *A. brasiliensis* ATCC 16404 | 392-1154 | Microbiologics Inc. | 5 | 31 Mar 2023 |
| *C. albicans* ATCC 10231 | 443-1166 | Microbiologics Inc. | 5 | 30 Jun 2022 |
| *E. coli* ATCC 8739 | 483-1052 | Microbiologics Inc. | 5 | 31 May 2022 |
| *P. aeruginosa* ATCC 9027 | 484-1263 | Microbiologics Inc. | 5 | 30 Jun 2022 |
| *S. aureus* ATCC 6538 | 485-964 | Microbiologics Inc. | 5 | 31 Aug 2022 |

Microbes *A. brasiliensis* and *C. albicans* were inoculated from their original ATCC strains to Sabouraud Dextrose Agar (SDA) slant and incubated at 20 °C for 48 h to create starter tubes for the preparation of microbial suspensions. The other microbes such as *E. coli*, *P. aeruginosa* and *S. aureus* were inoculated to Trypticase Soy Broth (TSB) tubes and incubated at 30–35 °C for 24 h. The strains were grown beforehand by a laboratory technician in Microbial QC laboratory.

From each cultivated starter tube, a small part of the microbial growth was inoculated again as described above. After the incubation, microbial growth was verified by perceiving cloudiness in the TSB tubes and visible colonies on the SDA slants.

After the incubation of cultivated starter tubes, microbes were diluted so that in 100 µl of the final dilution of microbial suspension, there were 10–100 colony forming units (CFU). Dilutions were made into buffered NaCl-Peptone solution. Microbial suspensions had to be used within 2 hours from the dilution for the suspensions to remain valid. For every set of validation, a new set of suspensions was made.

### 3.4.2 Preparation of rinsing solutions

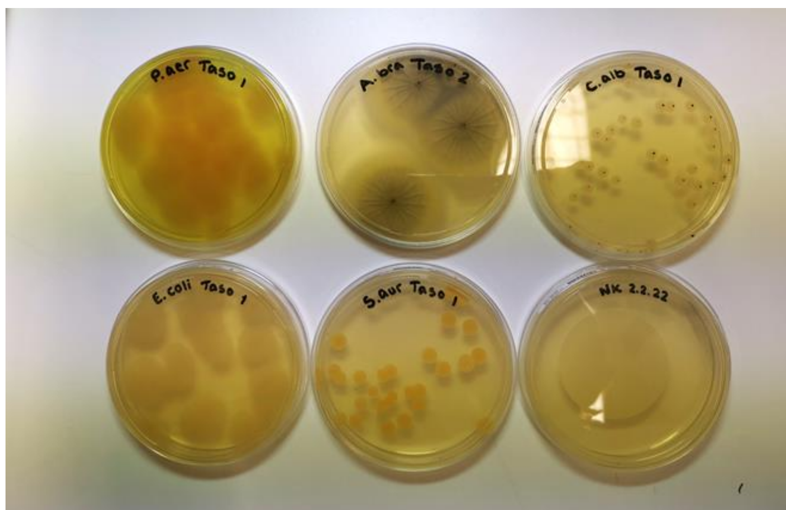
Rinsing solutions used in this analysis were 0.1% peptone water and 0.1% peptone water with 0.1% polysorbate 80. Both solutions were prepared from 10% peptone and 25% polysorbate 80 stock solutions. (Picture 3.) Dilutions were made into sterile water.

Picture 3. Polysorbate 80 (left) and peptone (right) stock solutions.

Total of 5 litres of 0.1% peptone water was prepared for rinsing each of the membranes three times 100 ml of the solution. Eight litres of 0.1% peptone water containing 0.1% polysorbate was made for rinsing the membranes five times 100 ml. All rinsing solutions were prepared on analysis day to verify the authenticity of the solutions. Solutions remained usable for 24 h.

### 3.5 Microbial control plates

Microbial control plates, showed below in picture 4., were made to show that in each challenge organism, there are required 10–100 cfu and that the dilution of challenge microbes was made successfully. If the amount of cfu in microbial control plates were out of specification, or they had significant difference in between, it would mean that the results of the filtration were not valid. The microbial growth of microbial control plates was compared with the microbial growth in the membrane, after the filtration of samples and recovery percentage was calculated.




Picture 4. A set of microbial control plates made for this validation.

Microbial control plates were prepared by the surface spread method. 100 µl of the challenge microbe was pipetted to a TSA agar and incubated for 5 days at 30–35 °C. Microbial control plates were made for every challenge microbe and in duplicate.

## 3.6 Product control and negative control

For every set of validation, a product and negative control were made. If contamination occurs in after the filtration, the source of the contaminant can be traced to either the sample or to the filtration method. The purpose of product control was to verify the purity of the sample solution. Product control was made by performing the filtration normally but excluding the adding of challenge organism to the final rinsing solution.

Negative control verifies the testing condition's authenticity. It is conducted by rinsing the PVDF membrane as in the method, but not filtering the sample and challenge organisms. Both the product and the negative control were made in duplicate to TSA agar and incubated 5 days at 30–35 °C. Any microbial growth in the product control or in the negative control will disqualify the validation.

# 4 RESULTS

Results for every set of validation with 0.04 µg/ml of levofloxacin, 0.054 µg/ml of BAC and 6.7 µg/ml of Deconex® CIP alpha-x are presented in appendices 1–6 on tables 5–10. Furthermore, the results of microbial control plates and microbial recoveries are presented. Microbial recoveries are calculated according to equation (1). In the calculation, an average value of microbial control plates is used. Average values were rounded up to a whole number. Original values from control plates, are shown in the brackets after the average value. Recoveries presented in appendices 1–6 are rounded up to a whole number. Recoveries that do not meet the acceptance criteria, are bolded.

## 4.1 Sample solution containing levofloxacin

On appendices 1–3, the results for the validation three sets of validation with 0.04 µg/ml of levofloxacin, are presented. Negative control and product control made from the water containing levofloxacin, showed no microbial growth. There was also no contamination in any of the plates. On table 2., an average value of recoveries of the results, is shown.

TABLE 2. Average recovery values of the recovery of sample containing levofloxacin.

| | | Challenge microbe | | | | |
|---|---|---|---|---|---|---|
| | | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
| Average recovery | 3x 100 ml | 72% | 93% | 107% | 86% | 70% |
| | 5x 100 ml | 62% | 74% | 156% | 107% | 73% |

All average recoveries from this sample solution are between 50–200%, which meets the acceptance criteria set for this validation. There is no significant difference between the different rinsing methods.

## 4.2 Sample solution containing cleaning agent and preservative

On appendices 4–6, the results for the validation session with 0,054 µg/ml of BAC and 6,7 µg/ml of Deconex® CIP alpha-x, are presented. Negative control and product control made from the water containing Deconex® CIP alpha-x and benzalkonium chloride, showed no microbial growth. There was a slight contamination in some of the plates of *S.aureus,* but the validation was not repeated. Contamination was noticed by different coloured colonies appearing on the plate. Below on table 3., an average value of recoveries of the results, is shown.

TABLE 3. Average recovery values of the recovery of sample containing Deconex® CIP alpha-x and benzalkonium chloride.

| | | Challenge microbe | | | | |
|---|---|---|---|---|---|---|
| | | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
| Average recovery | 3x 100 ml | 56% | 83% | 121% | 97% | 96% |
| | 5x 100 ml | 70% | 95% | 101% | 166% | 123% |

All average recoveries from this sample solution are between 50–200%, which meets the acceptance criteria set for this validation. There is no significant difference between the different rinsing methods.

# 5 DISCUSSION AND CONCLUSIONS

Validation results support the suitability of the membrane filtration method when analysing production rinsing waters according to Ph. Eur 2.6.12 and USP 61 when rinsing water containing a maximum residue of cleaning agent, preservative and API is filtered through a PVDF membrane, rinsed with 100 mL of rinsing fluid and incubated on Tryptic Soy Agar at 30–35 °C. This validation fulfils majority of the requirements set by GMP, Ph. Eur 2.6.12 and USP 61. A closure of all the requirements for this validation and their acceptance is shown in table 4.

TABLE 4. Closure of the validation's requirements.

| Requirements for the validation | 0,04 µg/ml of levofloxacin | 0,054 µg/ml of BAC and 6,7 µg/ml of Deconex® CIP alpha-x |
|---|---|---|
| Recovery of the sample plates is 50-200% from control plates | 48–174% | 46–160% |
| Every microbial control plate has 10–100 cfu | Yes | Yes |
| No growth in negative control | Yes | Yes |
| No growth in product control | Yes | Yes |

As in described earlier, the recovery of the challenge organisms meets the acceptance criteria when the recovery on the test plate is within 50 %-200 % of the microbial control plate. All but two of the microbial recoveries are within the limits of acceptance.

Recovery of 46% from the filtration of *A. brasiliensis* with the sample containing BAC and Deconex® CIP alpha-x is explainable by the possible mistakes on calculating the colonies from plates. *A. brasiliensis* forms large colonies rapidly and the possible smaller colonies might have left unnoticed. Mistakes such as erroneous pipetting of the mould suspension to the membrane can also explain the

results. Recovery of 48% from *S. aureus* with the sample containing levofloxacin can be justified by a fault in the preparation of *S. aureus* suspension or defective pipetting the suspension to the membrane. Nevertheless, no changes are made into the preparation of challenge microbes because of these two invalid results.

No microbial growth was detected in any negative control or product control. This denotes that the method itself does not have antimicrobial properties hampering the results. Extrinsic contamination was not occurring from any of the steps at this validation. Any microbial growth in negative control or product control would have rejected the analysis.

There were no relevant differences in the recovery percentage in between rinsing three times with 100ml of 0.1 % peptone water and rinsing five times with 100 ml of 0.1 % peptone water with 0.1 % polysorbate 80. Low recovery percent was noticed on both rinsing techniques: 46% recovery from *A. brasiliensis* was filtered five times with 100 ml of 0.1 % peptone water with 0.1 % polysorbate 80 and 48% recovery from *S. aureus* was filtered rinsing three times with 100 ml of 0.1 % peptone water. When no differences were perceived, there is no reason to rinse the membrane five times with 100 ml. Rinsing five times with 100 ml will only add material expenses and take time from the personnel working with the analysis.

Based on this validation, a method of analysis by the membrane filtration method is updated. From now on, the cellulose ester membrane is replaced with PVDF membrane for more reliable analysis. Membranes are rinsed 3x 100 ml of 0.1 % peptone water.

For further research, this method could be validated with other validation parameters than microbial recovery. When analysing the method with multiple parameters, the method will become more valid. For example, analysing robustness would make the method more reliable by recognizing different working methods, difference in interpretation of colonies and the characteristics of the sample matrix. (Hägg 2016, 22). All these occur, when more than one person is using this method for analysing production rinsing waters.

**REFERENCES**


Anderson, V. R. & Perry, C. M. 2008. Levofloxacin: A Review of its Use as a High-Dose, Short-Course Treatment for Bacterial Infection. Drugs (New York, N.Y.).

Clontz, L. 1998. Microbial limit and bioburden tests: validation approaches and global requirements. Buffalo Crove: Interpharm Press Inc.

Collin, F., Karkare, S. & Maxwell, A. 2011. Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. Applied Microbiology and Biotechnology. 92, 479–497.

Council of Europe. 2019. European Pharmacopoeia. Levofloxacin hemihydrate. 10th Edition. 3085–3086.

Council of Europe. 2021. European Pharmacopoeia. 2.6.12. Microbial Examination of Non-Sterile Products: Microbial Enumeration Tests. 10.3rd Edition. 4765–4769.

Deconex® CIP alpha-x. Brochure. Read on 17.3.2022. https://www.borer.ch/pdf_files.php?type=prodinfo&file=pdf_files/Pharma/english/Product% Borer Chemie AG. 2008.

Directive 2003/94/EC. Laying down the principles and guidelines of good manufacturing practice in respect of medicinal products for human use and investigational medicinal products for human use. Official Journal of the European Union. 8.10.2003. Read on 24.3.2022. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32003L0094&from=EN

EU Comission. 2020. GMP Annex 1. Manufacture of Sterile Products. Read on 8.3.2022. https://www.gmp-compliance.org/files/guidemgr/2020_annex1ps_sterile_medicinal_products_en.pdf

European Medicines Agency. Good Manufacturing Practice. Read on 24.3.2022. https://www.ema.europa.eu/en/human-regulatory/research-development/compliance/good-manufacturing-practice

Hägg, M. 2016. Validoinnin suunnittelun opas. VTT Technical Research Centre of Finland Ltd. Read on 3.4.2022. https://www.vttresearch.com/sites/default/files/pdf/technology/2016/T276.pdf

Johnson, D. S. & Li, J. J. 2007. The art of drug synthesis. Hoboken, New Jersey: John Wiley & Sons, Inc.

Lääkealan turvallisuus- ja kehittämiskeskus Fimea. Euroopan farmakopea. Read on 21.4.2022. https://www.fimea.fi/valvonta/lait_ja_ohjeet/euroopan_farmakopea

Merchel Piovesan Pereira, B. & Tagkopoulos, I. 2019. Benzalkonium Chlorides: Uses, Regulatory Status, and Microbial Resistance. Applied and environmental

microbiology, *85*(13), e00377-19. Read on 4.4.2022. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6581159/

Regulation 1119/2014. Amending Annex III to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for benzalkonium chloride and didecyldimethylammonium chloride in or on certain products. Official Journal of the European Union. 16.10.2014. Read on 4.4.2022. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32014R1119

Reese, R., Betts, R. & Gumustop, B. 2000. Handbook of Antibiotics. 3rd edition. Philadelphia: Lippincott Williams & Wilkins.

Sandle, T. 2015. Approaching Microbial Method Validation. Read on 18.4.2022. https://www.ivtnetwork.com/article/approaching-microbiological-method-validation

The United States Pharmacopeial Convention. 2021. United States Pharmacopeia. <61> Microbiological Examination of Nonsterile products: Microbial Enumeration tests.

**APPENDICES**

Appendix 1. 1st rinsing with sample containing levofloxacin.

TABLE 5. Results for 1st rinsing with sample containing levofloxacin.

| Challenge microbe | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
|---|---|---|---|---|---|
| CFU per plate | | | | | |
| Rinsing 3x 100 ml | 49 | 48 | 22 | 13 | 24 |
| Rinsing 5x 100 ml | 49 | 29 | 33 | 11 | 26 |
| Control plates | 85 (96;74) | 43 (41;45) | 19 (21;16) | 14 (20;8) | 35 (29;40) |
| Recovery for rinsing 3x 100 ml | 57% | 112% | 116% | 93% | 69% |
| Recovery for rinsing 5x 100 ml | 57% | 67% | 174% | 79% | 74% |

Appendix 2. 2nd rinsing with sample containing levofloxacin.

TABLE 6. Results for 2nd rinsing with sample containing levofloxacin.

| Challenge microbe | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
|---|---|---|---|---|---|
| CFU per plate | | | | | |
| Rinsing 3x 100 ml | 61 | 41 | 16 | 10 | 27 |
| Rinsing 5x 100 ml | 53 | 28 | 26 | 18 | 34 |
| Control plates | 85 (96;74) | 43 (41;45) | 19 (21;16) | 14 (20;8) | 35 (29;40) |
| Recovery for rinsing 3x 100 ml | 72% | 95% | 84% | 71% | 77% |
| Recovery for rinsing 5x 100 ml | 62% | 65% | 137% | 129% | 97% |

Appendix 3. 3rd rinsing with sample containing levofloxacin.

TABLE 7. Results for 3rd rinsing with sample containing levofloxacin.

| Challenge microbe | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
|---|---|---|---|---|---|
| CFU per plate | | | | | |
| Rinsing 3x 100 ml | 73 | 31 | 23 | 13 | 22 |
| Rinsing 5x 100 ml | 59 | 38 | 30 | 16 | 17 |
| Control plates | 85 (96;74) | 43 (41;45) | 19 (21;16) | 14 (20;8) | 35 (29;40) |
| Recovery for rinsing 3x 100 ml | 86% | 72% | 121% | 93% | 63% |
| Recovery for rinsing 5x 100 ml | 69% | 88% | 158% | 114% | **48%** |

Appendix 4. 1st rinsing with sample containing BAC and Deconex® CIP alpha-x.

TABLE 8. Results for 1st rinsing with sample containing BAC and Deconex® CIP alpha-x.

| Challenge microbe | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
|---|---|---|---|---|---|
| CFU per plate | | | | | |
| Rinsing 3x 100 ml | 47 | 54 | 36 | 25 | 29 |
| Rinsing 5x 100 ml | 65 | 66 | 24 | 40 | 25 |
| Control plates | 85 (96;74) | 66 (65;66) | 31 (28;34) | 25 (29;20) | 24 (25;22) |
| Recovery for rinsing 3x 100 ml | 55% | 82% | 116% | 100% | 120% |
| Recovery for rinsing 5x 100 ml | 76% | 100% | 77% | 160% | 104% |

Appendix 5. 2nd rinsing with sample containing BAC and Deconex® CIP alpha-x.

TABLE 9. Results for 2nd rinsing with sample containing BAC and Deconex® CIP alpha-x.

| Challenge microbe | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
|---|---|---|---|---|---|
| CFU per plate | | | | | |
| Rinsing 3x 100 ml | 56 | 54 | 28 | 23 | 36 |
| Rinsing 5x 100 ml | 62 | 58 | 30 | 14 | 36 |
| Control plates | 85 (96;74) | 66 (65;66) | 31 (28;34) | 25 (29;20) | 24 (25;22) |
| Recovery for rinsing 3x 100 ml | 66% | 82% | 90% | 92% | 156% |
| Recovery for rinsing 5x 100 ml | 73% | 87% | 97% | 178% | 156% |

Appendix 6. 3rd rinsing with sample containing BAC and Deconex® CIP alpha-x.

TABLE 10. Results for 3rd rinsing with sample containing BAC and Deconex® CIP alpha-x.

| Challenge microbe | *A. brasiliensis* | *C. albicans* | *E. coli* | *P. aeruginosa* | *S. aureus* |
|---|---|---|---|---|---|
| CFU per plate | | | | | |
| Rinsing 3x 100 ml | 39 | 56 | 47 | 25 | 35 |
| Rinsing 5x 100 ml | 51 | 63 | 40 | 40 | 26 |
| Control plates | 85 (96;74) | 66 (65;66) | 31 (28;34) | 25 (29;20) | 24 (25;22) |
| Recovery for rinsing 3x 100 ml | **46%** | 85% | 157% | 100% | 152% |
| Recovery for rinsing 5x 100 ml | 60% | 97% | 129% | 160% | 109% |