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Removal of bacterial growth inhibition of anticancer drugs by using complexation materials

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Abstract: In the context of batch production of cytotoxic drugs in hospital pharmacies with the need of sterility testing, the objective was to validate the use of Rapid Microbiological Method (RMM), and to develop adequate neutralization method in case of inhibition of bacterial growth. The potential microbiological growth inhibitory effect of three anticancer drugs (5 fluorouracil, irinotecan and oxaliplatin) selected for batch production was assessed on BacT/ALERT® system. Among cytotoxic drugs, only 5FU exhibited inhibitory effect on microbiological growth using rapid microbiological method. To counteract this effect our purpose was to use neutralizing agents complexing the drug i.e. activated carbon or ion exchange resins. The microbiological bactericidal concentration of 5FU was very low (1.10^-4 mg/mL) indicating the absolute need to neutralize the whole drug before sterility test. The complexation was validated by High Performance Liquid Chromatography control of the residual 5FU concentration in solution after the use of neutralizing agents. Only activated carbon was able to fully capture 5FU when previously diluted at 5 mg/mL. Conversely, the resins, in the condition of the study, were not able to fully capture 5FU whatever the dilution. The microbiological growth on BacT/ALERT® system after active carbon treatment was successfully confirmed with Staphylococcus aureus. Based on this validation results a method was then developed to routinely be able to perform sterility test of the batches produced and was confirmed on five microbiological species (i.e. S. aureus, Pseudomonas aeruginosa, Bacillus subtilis, Candida albicans, Aspergillus brasiliensis). Our work gives a new insight for considering sterility testing by rapid microbiological method even for drugs exhibiting inhibitory effect on microbiological growth.

Keywords: 5-fluorouracil; activated carbon; batch production; ion exchange resin; microbiological growth; sterility testing.

Introduction

Hospital cytotoxic compounding pharmacies had to face increasing needs without increasing means. One emerging solution was to develop dose banding [1] allowing batch preparations with longer term of storage. Considering microbiological quality control, based on European Good Manufacturing Practice [2] sterility tests must be implemented for batch release of injectable drugs. Moreover, one European expert consensus on physico-chemical stability studies on hospital preparations recommended the sterility testing in case of long term shelf-life preparation [3]. The recent international Guidance dedicated to the microbiological stability issue on assignment of microbiological shelf-life for hospital pharmacy aseptic preparations recommended to implement validation process for long term storage and sterility testing for routine batch release [4]. The reference filtration method provided by the European Pharmacopoeia for sterility testing [5] with 14 days incubation before the result is not adapted to the common few weeks shelf-life related to physico-chemical stability which can be assigned to hospital preparations. Rapid
Microbiology Methods (RMM) may be interesting alternative but must be validated, especially when the drug is able to inhibit microbiological growth. Thus, RMM have already been described as an alternative method for this sterility testing in hospital pharmacies [6, 7]. However, whatever the method used it is mandatory to validate the lack of inhibitory effect of the drug on microbiological growth. With regards to dose banding of cytotoxic drugs, it has been shown that with cytotoxic drug, microbiological growth inhibition is inconstantly found [8]. For example, Hamilton et al. [9] showed that mitomycin C, five fluorouracil (5FU) and methotrexate had significant inhibitory effect on the growth of 28 bacterial strains. On the other hand, the 17 remaining cytotoxic drugs tested showed a slight antimicrobial activity correlated to a Minimal Bactericidal Concentration (MBC) > 10 mg/L. Thus, microbial growth inhibition should be tested before performing a sterility test on hospital preparations, especially when using alternative method to Pharmacopeia such as RMM.

The Bactec® method has been previously evaluated [10] for this purpose and was not suitable for performing a sterility test on 5FU parenteral solutions. Besides, the BacT/ALERT® (BA) system another RMM has been already validated for sterility testing of parenteral nutrition [6] but was not able to allow the microbiological growth with 5FU parenteral solution. 5FU as model drug exhibiting inhibitory effect on microbiological growth was selected to find a suitable method of neutralization allowing to perform a sterility test when preparations are batch produced.

The objective of the present work is to assess the feasibility of use of RMM (i. e. BA) for sterility testing, for cytotoxic drugs prepared in batches (i. e. 5FU, oxaliplatin and irinotecan). 5FU was chosen as model cytotoxic drugs known to have inhibitory effect on microbiological growth [10] with MBC range between 0.5 and 8 μg/mL [9] and ones without inhibitory effects (i. e. oxaliplatin, irinotecan) as controls. For drugs with inhibitory effects on growth several methods are available. Firstly, according to the European Pharmacopoeia [5], it is possible to use dilution method to perform sterility control on products with antimicrobial activity. However, the low MBC of 5FU does not allow this method to be used [10]. Other Pharmacopeia method is the use of additional substances for neutralization or inactivation [5]. In our study, we assessed the feasibility of complexing systems to neutralize the inhibitory effect of five FU. We chose two complexing materials used in water treatment [11, 12]: activated carbon (AC) and ion exchange resins (IER). At the end, the goal was to study the feasibility of the designed method for routine sterility control of batches.

Materials and methods

Materials

The microbiological growth detection system used was BA system from Biomérieux (Marcy-l’Étoile, France) and the media bottles were FA plus (Aerobic) and FN plus (Anaerobic). These media bottles contain polymer beads of resin of undivulged nature from the provider (R1) and intended to absorb antibiotics in patients' blood. Additionally, four other IER: two IER have been kindly provided by Purolite company (Bala Cynwyd, USA), a weak cation exchange resin (C104E + called R2) and a strong cation exchange resin (CISOS called R3); and the two other IER were purchased from Sigma-Aldrich (Saint-Louis, USA), weak (Amberlyst A-21 called B4) and strong (Dowex 1X8-200 called R5) anion exchange resins. The characteristics of the resins are summarized in Table 1. AC was from Sigma-Aldrich (Saint-Louis, USA) and the particles' size was 100 mesh. 5FU 5000 mg/100 mL and Oxaliplatin 200 mg/40 mL were purchased from Accord Healthcare (Lille, France) and was used in 10 mL samples. Irinotecan was purchased from Medac (Lyon, France). Bacteria strains used were Staphylococcus aureus (S. aureus) ATCC6538, Pseudomonas aeruginosa (P. aeruginosa) ATCC9027, Bacillus subtilis (B. subtilis) ATTC6633, Candida albicans (C. albicans) ATTC10231, Aspergillus brasiliensis (A. brasiliensis) ATCC16404 and were purchased from Eurofins (Luxembourg).

Methods

Sterility test using RMM: The RMM system used was BA system which is an automated microbial detection system based on the colorimetric detection of CO₂ produced by growing microorganisms. The BA is using closed system media bottles: one for aerobic, and one for anaerobic bacteria in which a 10 mL sample to be analyzed was inoculated.

Each drug solution assessed for sterility test was incubated using BA system.

Results of microbiological growth after incubation are obtained between 24 h and 5 days depending on strains.

Assessment of potential inhibitory effect of drugs on bacterial growth using RMM system: The microbiological growth inhibitory effect was assessed for three anticancer drugs selected for batch production: 5FU, irinotecan and oxaliplatin. Dilutions of drug solutions were chosen to be in the range of final concentrations clinically used. 5FU solutions were assessed as undiluted (50 mg/mL) and diluted with NaCl 0.9% at 25, 15, 10, 5 mg/mL final concentrations. Oxaliplatin and irinotecan solutions were assessed at 0.4, 0.5, 0.6 and 0.7 mg/mL and 0.9, 1.3, 1.3, 1.5 mg/mL respectively.

Before addition of the 10 mL drug sample in BA media bottle, each bottle was inoculated with bacterial strains of S. aureus ≤100 Colony Forming Unit (CFU). Strains were obtained from frozen pellets dissolved in Tryptone soya from Sigma-Aldrich (Saint-Louis, USA) according to the manufacturer's recommendations, giving the final concentration of less than 100 CFU in accordance to the requirements of the European Pharmacopoeia [5]. Media bottles inoculated with strains and cytotoxic drug samples were incubated and analyzed by the detection system compared to controls. Negative control was inoculation of anticancer drug at its maximum concentration without
microbiological strain; positive control was inoculation of bacterial strains < 100 CFU without any anticancer drug.

**MBC determination:** MBC of anticancer drugs on *S. aureus* was determined using the liquid microdilution method. A range of concentrations in 96-well microplates and a control tube (control of the growth of the strain tested) were made. A fixed number of bacteria was seeded into each well. The reading was performed after 18 h of incubation at 37 °C, the MBC being the first dilution where bacterial growth was observed by a visual turbidity.

**Evaluation of the efficacy of neutralization method:** 5FU solutions inhibiting microbiological growth in RMM were assayed for neutralization method using AC or IER. Prior inoculation of the sample in BA media bottles, the drug solutions were put in contact with either AC or IER to allow complexation of the drug. The solution was then filtrated via vacuum filtration using various miscellaneous conditions (cytotoxic drug concentrations, complexation material concentrations and contact time). Each filtrate was assayed three times by High Performance Liquid Chromatography (HPLC). When anticancer drug was no longer detectable by HPLC method, the filtrate was incubated in BA medium bottles for assessment of microbiological growth in the same conditions as previously described. A conceptual summary of methods used is given in Figure 1.

To determine the optimum conditions of neutralization of 5FU solutions (50, 25, 10, 5 mg/mL) increasing concentrations of whether AC (30, 60, 90, 150, 300 mg/mL) or IER (200, 300, 400 mg/mL) were used and contact times between 5 min and maximum 24 h were tested. For each condition, samples were vacuum filtered through by 0.45 µm filter (Merck Millipore, Burlington, USA) and supernatant assayed for 5FU determination by HPLC (n = 3 per filtrate). The remaining percentage in comparison to the initial concentration was then determined. Choice of concentration of neutralizing agent was determined according to the quantity found in the BA bottle for IER and according to the maximum quantities that can be filtrated for AC. For all IER investigated the efficacy for complexing 5FU was unsatisfactory whatever the concentration tested, optimization of the conditions was therefore optimized only for AC. The need for two repeated vacuum filtrations were assessed on worst cases conditions i.e. 5FU undiluted (50 mg/mL) and average concentrations of AC (90 and 150 mg/mL) at two different days and on two different samples. These four samples were assayed three times by the HPLC method. Repeatability variance (σ²) was calculated.

**HPLC Chromatographic analysis:** The chromatographic analyses were performed in conditions given in Table 2. HPLC determination was performed in accordance to previously validated method [13]. Briefly, for 5FU, analysis was performed on a Kinetex XB-C18 column from Phenomenex (Torrance, USA) using a mobile phase composed of 80% MetOH and 20% HCOOH (0.1v/v) from Honeywell (Charlotte, USA), monitored at 266 nm (Table 2). For irinotecan and oxaliplatin chromatographic conditions are given in Table 2, using same HPLC device and column, but with Water 50% Acetonitrile 50% mobile phase and detection at 221 and 256 nm for irinotecan and oxaliplatin respectively. Summary of the validation of HPLC methods are given in Table 3.

| Table 1: | IER characteristics. |
|--------|---------------------|
| Resins | Reference           | Exchange  | Composition of skeleton | Functional grouping | Structure  |
| R1     | Bact/ALERT® system  | Unknown   | Unknown                 | Unknown             | Unknown    |
| R2     | Purolite C104E+     | Weak cation | Polyaacrylic acid      | Carboxylic acid    | Gel        |
| R3     | Purolite C150S      | Strong cation | Styren polysulfonate  | Sulfonic acid      | Macroporous |
| R4     | Amberlyst A-21      | Weak anion | Styren polysulfonate   | Alkyl amine        | Macroporous |
| R5     | Dowex 1X8-200       | Strong anion | Styren polysulfonate  | Trimethyl ammonium | Gel        |

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Figure 1: Summary of the method used for 5FU AC or IER neutralization.
For each agent, the quality control was assayed six times within 2 days. 5FU: 5-fluorouracil; QC: Quality control.
of 5FU, the AC concentrations tested and different contact times are given in Table 6. 5FU was nearly no longer detected for 25, 10 and 5 mg/mL 5FU solutions treated by 300, 150 and 90 mg/mL AC. The greater the AC concentration was used, the lower the 5FU concentration was remaining. The lower the 5FU initial concentration used, the more effective was the AC treatment. Changing the contact time has a significant impact on the efficacy of AC treatment statistically (p < 0.05) for 5 and 30 min contact time but it was not statistically different (p > 0.05) for more than 30 min contact time.

When it comes to R1, we measured 1.6 g of dry material per media bottle. The preliminary test (50 mg/mL 5FU and 150 mg/mL R1 with t = 5 min and t = 24 h) did not show a significant impact of contact time on remaining percentages of 5FU: 74.85% at 5 min and 76.20% at 24 h (p = 0.4219). That is why 5 min was the contact time chosen for all the experiments with R1. Treatment of 5FU samples (5–50 mg/mL) final concentrations with increasing R1 concentrations (200–500 mg/mL) are given in Table 5. 5FU final concentrations decreased from 10 to 25% but no significant differences were noticed with regards to 5FU initial dilutions (p = 0.4563). When R1 efficacy was assessed using BA media bottles (Table 7), a 20% decrease of the 5FU final concentration was noticed. This decrease corresponds to the initial 1/5 dilution applied when introducing samples in BA bottles. Concerning the four other IER, we evaluated several parameters that could influence the complexation.

The method designed for routine implementation gave satisfactory results (Table 9) showing constant microbiological growth of the five strains assessed for growth i.e. S. aureus, P. aeruginosa, B. subtilis, C. albicans, A. brasiliensis at 50 mg/mL initial 5FU concentration and 200 mg/mL AC. The detection time is longer than the positive control without AC, but all detection times were less than 48 h. The test for routine implementation was therefore validated.
The goal of our study was to find a feasible neutralization method for sterility testing of batches using RMM for cytotoxic drugs including the one which exhibited bacterial growth inhibitory effect. However, even for drug known as inhibitors, the antimicrobial activity of these preparations is inconstant at all concentrations, with all microorganisms and with all active substance [10], there is still a contamination risk of these preparations. The use of the BA system for the detection of microbiological growth was possible for oxaliplatin and irinotecan. The measured MBC confirmed the absence of inhibition at routine concentrations. Conversely, 5FU exhibited inhibitory activity on microbiological growth, which was not sufficiently countered by the dilution and the resin included in BA

### Discussion

The goal of our study was to find a feasible neutralization method for sterility testing of batches using RMM for cytotoxic drugs including the one which exhibited bacterial growth inhibitory effect. However, even for drug known as inhibitors, the antimicrobial activity of these preparations is inconstant at all concentrations, with all microorganisms and with all active substance [10], there is still a contamination risk of these preparations. The use of the BA system for the detection of microbiological growth was possible for oxaliplatin and irinotecan. The measured MBC confirmed the absence of inhibition at routine concentrations. Conversely, 5FU exhibited inhibitory activity on microbiological growth, which was not sufficiently countered by the dilution and the resin included in BA

### Table 4: Bacterial detection of contaminated sample with BacT/ALERT® system.

| Anticancer drug | Concentration before incorporation into BacT/ALERT® bottles (mg/mL) | Bacterial contamination (CFU) | Bacterial detection by BacT/ALERT® system |
|-----------------|---------------------------------------------------------------|-------------------------------|------------------------------------------|
| None (NaCl)     | 0                                                             | <100                          | (+)                                      |
| 5FU             | 50                                                           | 0                             | (-)                                      |
|                 | 50                                                           | <100                          | (-)                                      |
|                 | 25                                                           | <100                          | (-)                                      |
|                 | 15                                                           | <100                          | (-)                                      |
|                 | 10                                                           | <100                          | (-)                                      |
|                 | 5                                                            | <100                          | (-)                                      |
| Oxaliplatin     | 0.7                                                          | 0                             | (-)                                      |
|                 | 0.7                                                          | <100                          | (+)                                      |
|                 | 0.6                                                          | <100                          | (+)                                      |
|                 | 0.5                                                          | <100                          | (+)                                      |
|                 | 0.4                                                          | <100                          | (+)                                      |
| Irinotecan      | 1.5                                                          | 0                             | (-)                                      |
|                 | 1.3                                                          | <100                          | (+)                                      |
|                 | 1.1                                                          | <100                          | (+)                                      |
|                 | 0.9                                                          | <100                          | (+)                                      |

(+) microbiological growth; (-) lack of microbiological growth.

### Table 5: Evaluation of 5FU complexation by IERs.

| 5FU concentration (mg/mL) | Resin concentration (mg/mL) | Contact time | Remaining percentages of 5FU from initial concentration |
|---------------------------|-----------------------------|--------------|--------------------------------------------------------|
|                           |                             |              | R1 | R2 | R3 | R4 | R5 |
| 50                        | 200                         | 5 min        | 87.5 ± 0.15 | 73.1 ± 0.31 | 91.1 ± 0.20 | 83.6 ± 0.05 | 60.6 ± 0.14 |
|                           |                             | 24 h         | ND | 21.1 ± 0.05  | 91.9 ± 0.22  | 79.4 ± 0.15  | 50.1 ± 0.03  |
| 300                       | 5 min                       | 84.2 ± 0.04  | 36.1 ± 0.28 | 82.9 ± 0.28  | 74.7 ± 0.06  | ND            |
| 400                       | 5 min                       | 79.4 ± 0.10  | 41.8 ± 0.05 | 82.9 ± 0.07  | 68.4 ± 0.05  | ND            |
| 500                       | 5 min                       | 78.7 ± 0.03  | 31.3 ± 0.05 | 86.6 ± 2.43  | 62.5 ± 0.06  | 34.4 ± 0.04  |
| 25                        | 200                         | 89.3 ± 0.33  | 71.1 ± 0.07 | ND | 72.2 ± 0.24  | 49.0 ± 0.04  |
|                           |                             | 24 h         | ND | 82.7 ± 0.03  | 66.9 ± 0.39  | ND | ND |
| 300                       | 5 min                       | 78.0 ± 0.02  | 81.3 ± 0.29 | ND | ND | ND |
| 400                       | 5 min                       | 73.2 ± 0.04  | 77.7 ± 0.30 | ND | ND | ND |
| 10                        | 200                         | 84.3 ± 0.54  | 84.6 ± 0.29 | ND | 68.0 ± 0.21  | 40.1 ± 0.07  |
|                           |                             | 24 h         | ND | 78.0 ± 0.02  | 66.9 ± 0.39  | 60.2 ± 0.06  |
| 300                       | 5 min                       | 84.0 ± 0.29  | 95.6 ± 0.06 | 69.3 ± 0.39  | 40.2 ± 0.06  |
| 400                       | 5 min                       | 73.8 ± 0.22  | 95.6 ± 0.51 | 63.0 ± 0.39  | 39.2 ± 0.10  |
| 5                         | 200                         | 86.6 ± 0.29  | 84.6 ± 0.29 | 95.6 ± 0.06 | 66.9 ± 0.39  | 40.2 ± 0.06  |
|                           |                             | 24 h         | ND | 73.8 ± 0.22  | 95.6 ± 0.51  | 63.0 ± 0.39  | 39.2 ± 0.10  |
| 300                       | 5 min                       | 76.0 ± 0.08  | 77.5 ± 0.08 | 90.7 ± 0.25  | 52.4 ± 0.06  | ND |
| 400                       | 5 min                       | 72.9 ± 0.09  | 72.9 ± 0.09 | 87.0 ± 0.09  | 43.0 ± 0.05  | ND |
| 500                       | 5 min                       | 73.8 ± 0.12  | 73.8 ± 0.12 | 89.4 ± 1.15  | 36.3 ± 0.06  | 47.2 ± 0.06  |

*White precipitate.*
Table 6: Contact time evaluation for complexation by activated carbon via vacuum filtration.

| SFU concentration (mg/mL) | AC concentration (mg/mL) | Remaining percentage of SFU from initial concentration |
|---------------------------|--------------------------|------------------------------------------------------|
|                           |                          | t = 5 min | t = 30 min | t = 1 h | t = 24 h |
| 50                        | 30                       | 98.6 ± 0.15 | 77.2 ± 0.16 | 84.3 ± 0.08 | 77.5 ± 0.18 |
|                           | 60                       | 70.6 ± 0.11 | 66.3 ± 0.09 | 67.5 ± 0.18 | 68.0 ± 0.06 |
|                           | 90                       | 73.7 ± 0.11 | 53.5 ± 0.06 | 58.9 ± 0.23 | 61.9 ± 0.05 |
|                           | 150                      | 46.1 ± 0.34 | 39.3 ± 0.05 | 41.0 ± 0.03 | 39.4 ± 0.05 |
|                           | 200                      | 31.0 ± 0.02 | ND          | ND        | ND        |
|                           | 300                      | 28.3 ± 0.08 | 8.9 ± 0.01  | 12.4 ± 0.01 | 8.0 ± 0.01 |
| 25                        | 30                       | 78.6 ± 0.02 | 89.8 ± 0.10 | 78.0 ± 0.06 | 75.3 ± 0.06 |
|                           | 60                       | 58.0 ± 0.09 | 61.5 ± 0.12 | 56.6 ± 0.11 | 55.7 ± 0.11 |
|                           | 90                       | 43.0 ± 0.01 | 41.9 ± 0.05 | 42.5 ± 0.05 | 38.4 ± 0.00 |
|                           | 150                      | 19.0 ± 0.05 | 16.0 ± 0.11 | 18.2 ± 0.03 | 14.5 ± 0.05 |
|                           | 200                      | 9.0 ± 0.02  | ND          | ND        | ND        |
|                           | 300                      | 1.0 ± 0.01  | 1.1 ± 0.00  | 1.2 ± 0.01 | <LOQ      |
| 10                        | 30                       | 44.8 ± 0.07 | 54.9 ± 0.10 | 56.9 ± 0.13 | 57.8 ± 0.30 |
|                           | 60                       | 28.5 ± 0.10 | 25.4 ± 0.03 | 29.8 ± 0.03 | 27.3 ± 0.08 |
|                           | 90                       | 10.4 ± 0.02 | 9.2 ± 0.02  | 12.2 ± 0.02 | 8.9 ± 0.06 |
|                           | 150                      | 1.3 ± 0.01  | 0.3 ± 0.01  | 0.6 ± 0.01 | 0.2 ± 0.01 |
|                           | 300                      | <LOQ        | <LOQ        | <LOQ      | <LOQ      |
| 5                         | 30                       | 37.9 ± 0.02 | 40.4 ± 0.56 | 25.3 ± 0.02 | 30.1 ± 0.11 |
|                           | 60                       | 6.6 ± 0.02  | 4.9 ± 0.01  | 2.7 ± 0.02 | 4.7 ± 0.03 |
|                           | 90                       | <LOQ        | <LOQ        | <LOQ      | <LOQ      |
|                           | 150                      | <LOQ        | <LOQ        | <LOQ      | <LOQ      |
|                           | 300                      | <LOQ        | <LOQ        | <LOQ      | <LOQ      |

Results are mean ± SD of three determinations; LOQ = limit of quantification; ND = Not Determined.

System due to the very low MBC. The MBC assessed in our study (1 × 10^-6 mg/mL) was in accordance with previous published data (0.5–8 μg/mL [8]). This very low MBC explains the need for a very important expected complexation yield of 99.9998% for a 50 mg/mL SFU concentration.

For removal bacterial growth inhibition, specific inhibition or degradation of the given drug could be effective, however the main inconvenient is the need to develop specific method for each drug.

In our study, and for 5 FU, we chose complexation materials that are already used in water treatment: AC and IER. Haddad et al. [15] obtained more than 80% complexation of drugs (e. g. fluoxetine, diclofenac) using anionic or cationic resins. This neutralization method has the advantage of being non-specific to one given drug but depends on ionic charge of the drug. The effectiveness of the complexation may however depend on the attractiveness between the drug and the complexation material. IER [15] mechanism is based on their capacity to attract counter charged ionic solution. Ions enter the resin until Donnan’s equilibrium, then active groups dissociate and exchange. When there is unequal electrochemical potential, the charged IER tends to retain ions of opposite signs. The Donnan’s equilibrium is thus created and is characterized by a non-zero membrane potential difference (Figure 3).

Table 7: SFU remaining percentage after R1 treatment in BacT/ALERT® media bottles.

| Initial theoretical SFU concentration (mg/mL) | Theoretical SFU concentration after BacT/ALERT® bottle dilution (mg/mL) | Average remaining percentages of SFU |
|---------------------------------------------|------------------------------------------------------------------------|-------------------------------------|
|                                             | t = 5 min | t = 24 h |
| 50                                          | 10        | 106.8 ± 1.42 | 96.4 ± 0.65 |
| 25                                          | 5         | 98.2 ± 0.25  | 93.3 ± 0.10 |
| 10                                          | 2         | 104.0 ± 5.96 | 107.6 ± 0.25 |
| 5                                           | 1         | 113.5 ± 4.38 | 104.9 ± 0.60 |

Results are Mean ± SD of three determinations; LOQ = limit of quantification; ND = Not Determined.
The BA bottles, usually used for blood detection of microorganisms, contain absorbing polymeric beads that allow the neutralization of antibiotics potentially circulating in patients’ blood. The action of these beads, whose composition was not provided by the manufacturer, did not allow enough complexation of 5FU to allow bacterial growth. Our study confirmed that IER present in BA bottles (R1) are not sufficient to neutralize the 5FU microbiological inhibitory effects. Even after extraction from media bottles, we found that their action was not sufficient to fully capture 5FU. The only slight effect we found can be attributed to the final 1/5 dilution. Better results were found for antibiotics [7]. For example, Mitteregger et al. [7] evaluating the bacterial growth detection time in patient’s blood after antibiotic administration showed that antibiotics such as clindamycine, gentamicin and vancomycin present in patient’s serum were fully complexed by BA IER. But it should be noticed that blood concentration is at much lower level than in the preparation. Another study, assessing the efficacy of microbiological growth with another RMM system Bactec® on cytotoxic preparations, found efficiency for several cytotoxic drugs except for 5FU and gemcitabine.

Table 8: Bacterial growth from 5 FU solutions after AC treatment. Number of measures = 3; Format = m ± σ with m = percentage average and σ = standard deviation.

| Concentration of 5FU before incorporation into BacT/ALERT® bottles (mg/mL) | AC concentration (mg/mL) | Bacterial contamination (CFU) | Bacterial detection by the BacT/ALERT® system |
|---|---|---|---|
| 25 | 300 | <100 | (+) |
| 25 | 300 | <100 | (+) |
| 25 | 300 | <100 | (-) |
| 10 | 150 | <100 | (+) |
| 10 | 150 | <100 | (+) |
| 10 | 150 | <100 | (-) |
| 0 | 0 | <100 | (+) |

Table 9: Bacterial growth from 5 FU solutions using new designed routine sterility test combining AC pretreatment & BA.

| 5FU concentration (mg/mL) | AC concentration (mg/mL) | Sample quantity | Initial bacterial contamination (CFU) | Bacterial detection by BacT/ALERT® system | Average of detection time |
|---|---|---|---|---|---|
| 0 | 200 | 3 | *Staphylococcus aureus* <100 | (+) | Aerobic: 7.3 h Anaerobic: 9.2 h |
| 50 | 200 | 3 | *Pseudomonas aeruginosa* <100 | (+) | Aerobic: 16.2 h Anaerobic: 15.2 h |
| 0 | 200 | 3 | *Bacillus subtilis* <100 | (+) | Aerobic: 33.9 h Anaerobic: 12.9 h |
| 50 | 200 | 3 | *Candida albicans* <100 | (+) | Aerobic: 36.1 h Anaerobic: 11.5 h |
| 0 | 200 | 3 | *Aspergillus brasiliensis* <100 | (+) | Aerobic: 19.4 h Anaerobic: 12.5 h |
| 50 | 200 | 3 | *Candida albicans* <100 | (+) | Aerobic: 34.4 h Anaerobic: 18.6 h |
| 0 | 200 | 3 | *Aspergillus brasiliensis* <100 | (+) | Aerobic: 8.2 h Anaerobic: 11.2 h |
| 50 | 200 | 3 | *Aspergillus brasiliensis* <100 | (+) | Aerobic: 23.7 h Anaerobic: 17.6 h |

(+) microbiological growth (-) lack of microbiological growth.
confirming [10] the difficulty to sterility test 5 FU solutions by RMM.

When it comes to the other resins investigated in our study, anion exchange resins (R4 and R5) showed better complexation results for 5FU due to their basic structure (pKa = 8.02 [16]). In fact, the 5FU pH in solution is about 9 (pH > pKa), 5FU is therefore in basic form (A-) and is attracted to the positive charges of anion exchange resins structure. In our experiments, we found an exception for 50 mg/mL concentration treated by R2 (e.g. 31.32% for 200 mg/mL AC at 24 h), a white precipitate was observed. This reaction could be explained by an esterification reaction between 5FU and R2 carboxylic group.

Besides resins, we investigated AC as another complexation material active by tar removal process [17].

AC exhibits complex groups (consisting mainly of carbon atoms) on its surface and an extremely porous structure. These characteristics give it an important contact surface and adsorbent properties. It is ideally suited to adsorb organic compounds, especially species containing aromatic functions [18]. Depending on its particle size, it can retain particles from 1 to 10 nm, which is advantageously used for water treatment to retain chlorine, pesticides and organic compounds [19]. In water treatment only trace contaminants are captured, considering preparations the amount to be neutralized is very high. When it comes to drug high concentration AC was found to be effective for mitomycin C complexation. Experimentally, for drug targeting, AC (50 mg/mL) has been successfully used to complex more than 99% mitomycin C (1 mg/mL) [20].

In addition, AC is not favoring bacteria adsorption and is a favorable medium for bacterial growth [19]. In worst case conditions when 5FU was not totally complexed by AC, microbial growth was observed in 2 out of 3 samples assessed for sterility test by BA. To ensure the 100% growth for routine method, we decided to combine two methods recommended by European Pharmacopoeia [5]: dilution and neutralization of the drug. The conditions allowing 100 % growth on all species investigated were 1/10 dilution of the drug with complexation material in the syringe at the maximum AC quantity (i.e., 2 g) for properly collecting the filtrate. Even the detection time is longer, it stays within 48 h which is acceptable for quarantine before batch release. Tests must be performed on other cytotoxic agents with recognized inhibitory properties (e.g., mitomycin, methotrexate). This designed method for routine sterility testing would allow the batch production of 5FU and a longer term storage e.g., 28 days for 8, 10 and 50 mg/mL concentrations when considering the physicochemical stability studies results [21–23]. RMM using BA will be directly usable for oxaliplatin and irinotecan for sterility test of batches.

**Conclusion**

Our study enabled to use RMM for sterility testing of three cytotoxic drugs batch produced (irinotecan, oxaliplatin and 5FU). Even when the drug exhibited inhibitor effect on microbiological growth, the neutralization method associated with AC pretreatment allowed the sterility test.

The new designed method is currently been validated for other drugs exhibiting inhibiting effect on bacterial growth in order to determine its large applicability. In parallel we currently work on the development of a specific device which will help the transfer of this new technology for sterility testing of batch production in hospital pharmacies.

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