INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES
[ISSN: 0975-4725; CODEN(USA): IJPS00]
Journal Homepage: https://www.ijpsjournal.com

OPEN ACCESS

Research Article

Culture-Medium Composition Modulates the Efficacy of a Benzalkonium Chloride–Isopropyl Alcohol Disinfectant: Development, Evaluation and Contact-Time Remediation for Pharmaceutical Microbiology Laboratories

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ARTICLE INFO

Published: 27 Nov 2026
Keywords:
disinfectant, benzalkonium chloride, isopropyl alcohol, culture media, MacConkey agar, contact time, Plackett-Burman design, pharmaceutical microbiology
DOI:
10.5281/zenodo.24581949

ABSTRACT

Background: Disinfectants used in pharmaceutical facilities are normally validated on inert surfaces such as stainless steel and glass. Surfaces contaminated with culture media are a realistic but poorly characterised challenge, because media components can interact with the active agent. Methods: A benzalkonium chloride (BAC) and isopropyl alcohol (IPA) disinfectant was developed using a 12-run Plackett-Burman screening design and characterised physicochemically. Minimum inhibitory and bactericidal/fungicidal concentrations, time-kill kinetics, sporicidal activity and ICH Q1A (R2) stability (24 months long-term, 6 months accelerated) were determined. Efficacy against reference strains was measured on tryptic soy agar (TSA), Sabouraud dextrose agar (SDA), brain heart infusion (BHI) agar, MacConkey agar and nutrient agar (NA) using a quantitative agar-surface test (n = 9 per condition) and analysed by two-way ANOVA. Results: IPA, BAC and EDTA were the significant formulation factors, and the optimised product (0.1% BAC, 70% IPA) met all release specifications. At 5 minutes it met the pharmacopoeial criteria on TSA, BHI and NA. On MacConkey agar, log10 reductions fell to 3.8 for *Escherichia coli* and 3.5 for *Pseudomonas aeruginosa*, 1.6 log10 below TSA (Cohen's $d > 4$; medium × organism interaction $p < 0.001$). Fungal reductions were modestly lower on acidic SDA. Extending contact time to 10 minutes restored compliance on MacConkey agar. The product was not sporicidal at use concentration (2.1 log10 in 120 minutes) and remained within specification for 24 months. Conclusions: Culture-medium composition can reduce the efficacy of a cationic disinfectant, most plausibly through ion-pair formation with bile salts. Disinfectant validation should include relevant media, and laboratory procedures should specify a 10-minute contact time where media contamination is likely.

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

INTRODUCTION

Control of microbial contamination is central to pharmaceutical manufacturing and quality control, and surface disinfection is one of the main barriers in a contamination control strategy [1,14]. Quaternary ammonium compounds (QACs), of which benzalkonium chloride (BAC) is the most widely used, are valued for their broad antibacterial and antifungal activity, low volatility and compatibility with surfaces, and they are frequently combined with alcohols to speed up the kill [1,2]. BAC acts by adsorbing to the negatively charged cell envelope, disrupting membrane integrity and causing leakage of cytoplasmic contents [1,20]. Gram-negative bacteria, particularly *P. aeruginosa*, and fungal spores are intrinsically less susceptible, and reduced susceptibility can also be acquired [21,22,25], which has stimulated interest in alternative antimicrobial chemotypes such as natural-product hybrids [26].

Disinfectant efficacy is conventionally assessed in suspension tests or carrier tests on inert, non-porous surfaces, and many pharmacopoeial and standards-based methods follow this pattern [9,13]. Microbiology laboratories, however, handle large volumes of agar media, and spills and splashes of media are common. Media are complex: they contain peptones, carbohydrates, selective agents and, in the case of MacConkey agar, bile salts. Bile salts are anionic, strongly surface-active molecules [4], and anionic surfactants form ion pairs and mixed aggregates with cationic surfactants that have much lower surface activity than either component [5,6]. It is therefore plausible that a cationic biocide such as BAC could be partly inactivated by contact with some media, yet we found no published systematic comparison of QAC-alcohol disinfectant efficacy across the routine media of a pharmaceutical microbiology laboratory.

The present study had four aims: (i) to develop and characterise a BAC-IPA disinfectant using a screening design; (ii) to determine its inhibitory, bactericidal and kinetic profile; (iii) to compare its efficacy on five culture media and test whether medium composition alters the outcome; and (iv) to evaluate sporicidal activity, stability and material compatibility so that the findings could be translated into practical disinfection procedures.

MATERIALS AND METHODS

2.1 Formulation development and characterisation

Six formulation variables were screened in a 12-run Plackett-Burman design [11]: BAC concentration (0.05% or 0.2%), IPA concentration (50% or 80%), EDTA (0.01% or 0.1%), polysorbate 80 (0.1% or 0.5%), NaCl (0.1% or 0.9%) and pH (5.5 or 7.5). Main effects were calculated as the difference between mean responses at the high and low levels, and significance was judged at the 5% level. Responses were the zones of inhibition against *Staphylococcus aureus* and *P. aeruginosa*. The optimised formulation was characterised for appearance, pH, viscosity, surface tension, BAC content, IPA content (gas chromatography), zeta potential and sterility, and by FTIR spectroscopy.

2.2 Test organisms and media

Reference strains were *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404 and *Bacillus subtilis* ATCC 6633 (vegetative cells and spores). Bacterial suspensions were adjusted to 0.5 McFarland standard; fungal spore suspensions were adjusted to approximately 10^7 spores/mL. Five media were tested: TSA (reference medium), SDA, BHI agar, MacConkey agar and NA. Each lot of medium was subjected to growth-promotion testing, and plates were checked for sterility, fill depth and pH.

2.3 MIC, MBC/MFC and time-kill studies

MIC was determined by microbroth dilution following CLSI M07-A10 [10], using two-fold dilutions from 0.002% to 1.0% and an inoculum of 5×10^5 CFU/mL, each in triplicate on three days. MBC/MFC was the lowest concentration giving at least a 3 log10 reduction. Time-kill studies were run at the use concentration (0.1% BAC / 70% IPA) with sampling at 1, 2, 5, 10 and 30 minutes at 21 ± 2 °C. Samples were transferred immediately into D/E Neutralizing Broth.

2.4 Efficacy across culture media

The primary efficacy test was an agar-surface quantitative test adapted from EN 1276 and EN 1650 [9]. Plates were dried at 37 °C for 30 minutes; 0.1 mL of organism suspension (10^7–10^8 CFU/mL in PBS with

0.3 g/L bovine serum albumin as clean-conditions interfering substance) was spread on the agar surface and dried for 5 minutes. One millilitre of disinfectant at use concentration was spread over the surface for 5 or 10 minutes, after which plates were flooded with 5 mL D/E Neutralizing Broth, scraped, serially diluted and plated on non-selective medium. Log10 reduction was calculated as log10 (N0/Nt), where N0 is the count of the viability control. Acceptance criteria were at least 5 log10 for bacteria and at least 4 log10 for fungi [13]. The neutralizer was validated against four control groups (viability, neutralizer toxicity, neutralization efficacy and diluent), following the recovery principles of Dey and Engley and of Sutton and colleagues [7,8], with recovery of at least 50% of the viability control as the acceptance criterion. Each combination was tested in triplicate on three independent days (n = 9). A supplementary experiment measured the MIC of BAC against *E. coli* with and without 1.5 g/L deoxycholate.

2.5 Sporicidal activity, stability and compatibility

Sporicidal activity was assessed against *B. subtilis* spores (10^8 spores/mL) at 0.1%, 0.25% and 0.5% BAC-equivalent concentration with contact times of 5 to 120 minutes. Stability followed ICH Q1A (R2) [12]: 24 months at 25 °C/60% RH and 6 months at 40 °C/75% RH, monitoring appearance, pH, BAC content, IPA content, zone of inhibition and microbial contamination. Compatibility was graded on four facility materials.

2.6 Statistical analysis

Data were analysed in SPSS v27 using two-way ANOVA with Tukey post-hoc testing ($p < 0.05$). Differences from TSA were also expressed with 95% confidence intervals, Welch's test and Cohen's d from the summary statistics. Values reported as not detected were treated as censored at 6.0 log10.

RESULTS

3.1 Formulation development and characterisation

The screening design identified IPA concentration ($p = 0.001$), BAC concentration ($p = 0.003$) and EDTA concentration ($p = 0.042$) as the significant factors, whereas polysorbate 80, NaCl and pH had no significant main effect within the tested ranges. The optimised formulation used 0.1% BAC and 70% IPA rather than the high levels of 0.2% and 80%, because higher BAC raises the risk of surface residues and of cationic interaction with anionic soils, and 80% IPA increases flammability and evaporation. The EDTA effect is consistent with its known ability to destabilise the Gram-negative outer membrane [15,16].

The optimised product was a clear, colourless to pale yellow liquid with pH 6.95 ± 0.08, viscosity 1.98 ± 0.12 cP, surface tension 29.4 ± 0.6 mN/m, BAC content 98.6 ± 1.2% of label claim, IPA content 69.8 ± 0.4% v/v and zeta potential +32.4 ± 2.1 mV. It was sterile, and all attributes met their specifications. FTIR showed the expected bands of the components with no new bands.

3.2 MIC, MBC/MFC and time-kill kinetics

All MBC/MIC ratios were 2.5 or lower, indicating bactericidal and fungicidal rather than inhibitory activity (Table 1). The MIC rose from 0.0125% for *S. aureus* to 0.05% for *P. aeruginosa* and 0.1% for *A. brasiliensis*, in line with the known hierarchy of susceptibility [1]. At the use concentration of 0.1% BAC, the margin over the MIC was eight-fold for *S. aureus* but only two-fold for *P. aeruginosa* and *C. albicans*, and one-fold for *A. brasiliensis*.

Table 1: MIC and MBC/MFC of the Formulation (% BAC Equivalent)

Organism	MIC	MBC / MFC	MBC/MIC	Classification
S. aureus ATCC 6538	0.0125	0.025	2	Bactericidal
E. coli ATCC 8739	0.025	0.05	2	Bactericidal
P. aeruginosa ATCC 9027	0.05	0.1	2	Bactericidal
C. albicans ATCC 10231	0.05	0.1	2	Fungicidal
A. brasiliensis ATCC 16404	0.1	0.25	2.5	Fungicidal
B. subtilis ATCC 6633 (vegetative)	0.025	0.05	2	Bactericidal

In time-kill experiments, *S. aureus* and vegetative *B. subtilis* reached at least 5 log10 reduction within 2 to 5 minutes. *E. coli* reached 5.8 ± 0.3 and *P. aeruginosa* 5.2 ± 0.2 log10 at 5 minutes, and all organisms reached the limit of detection (6.0 log10) by 10 minutes. *P. aeruginosa* showed the slowest kinetics (2.1 ± 0.4 log10 at 1 minute).

3.3 Efficacy across culture media

Two-way ANOVA showed significant effects of medium ($F(4,70) = 18.43$, $p < 0.001$), organism ($F(4,70) = 22.18$, $p < 0.001$) and the medium × organism interaction ($F(16,70) = 4.32$, $p < 0.001$). Results at 5 minutes are summarised in Table 2. On TSA, BHI and NA all three bacterial species met the 5 log10 criterion. On MacConkey agar, *S. aureus* still complied (5.3 ± 0.3), but *E. coli* (3.8 ± 0.4) and *P. aeruginosa* (3.5 ± 0.3) did not.

Table 2: Log10 Reduction (Mean ± SD, N = 9) at 5-Minute Contact Time

Organism	TSA	SDA	BHI	MacConkey	NA
S. aureus	5.7 ± 0.2	not tested	5.5 ± 0.2	5.3 ± 0.3	5.6 ± 0.2
E. coli	5.4 ± 0.3	not tested	5.2 ± 0.3	3.8 ± 0.4*	5.3 ± 0.2
P. aeruginosa	5.1 ± 0.2	not tested	5.0 ± 0.3	3.5 ± 0.3*	5.1 ± 0.3
C. albicans	4.8 ± 0.2	4.3 ± 0.3*	not tested	not tested	not tested
A. brasiliensis	4.1 ± 0.3	3.8 ± 0.2*	not tested	not tested	not tested

*Significantly lower than TSA, $p < 0.05$. Selective and fungal media were paired only with the organisms for which they are relevant.

The difference from TSA was 1.60 log10 for both *E. coli* (95% CI ± 0.36, Cohen's d = 4.53) and *P. aeruginosa* (± 0.26, d = 6.28) on MacConkey agar (Welch $p < 0.001$ for both). The *S. aureus* difference on MacConkey agar was small (0.40 log10), the fungal differences on SDA were moderate (0.50 log10 for *C. albicans*, 0.30 for *A. brasiliensis*), and BHI and NA did not differ from TSA. *A. brasiliensis* on SDA (3.8 log10) fell just below the 4 log10 fungal criterion, whereas *C. albicans* (4.3 log10) met it.

Extending the contact time from 5 to 10 minutes on MacConkey agar raised the reduction of *E. coli* from 3.8 to 5.4 and of *P. aeruginosa* from 3.5 to 5.1 log10, restoring compliance; *S. aureus* reached the detection limit. For *P. aeruginosa* the margin above the criterion was only 0.1 log10. Ten-minute data were tabulated for TSA and MacConkey agar, where every organism met the criterion.

In the supplementary experiment, the MIC of BAC against *E. coli* increased roughly ten-fold in the presence of 1.5 g/L deoxycholate.

3.4 Sporicidal activity, stability and compatibility

The formulation was not sporicidal at its use concentration: 0.1% BAC gave 0.3 ± 0.1 log10 reduction of *B. subtilis* spores at 5 minutes and 2.1 ± 0.2 log10 at 120 minutes. Activity rose with concentration (3.1 ± 0.2 log10 at 0.25% and 5.2 ± 0.4 log10 at 0.5% after 120 minutes). Linear apparent rates were 0.0149, 0.0213 and 0.0368 log10/min at 0.1%, 0.25% and 0.5% BAC, giving a concentration exponent of 0.55 ($R^2 = 0.96$).

All stability parameters remained within specification. BAC content declined from 98.6% to 95.4% of label claim over 24 months at 25 °C/60% RH (specification 90–110%), IPA content from 69.8% to 68.5% v/v, pH from 6.95 to 6.85, and the zone of inhibition against *S. aureus* from 22.1 to 21.2 mm (specification at least 20 mm). No microbial growth was detected at any time point. The 6-month accelerated data showed a smaller change (BAC content 96.8%), supporting assignment of a 24-month shelf life. Material compatibility testing found the product compatible with the facility materials tested, with the exception that prolonged contact with epoxy resin flooring should be avoided.

DISCUSSION

The principal finding of this study is that the efficacy of a BAC-IPA disinfectant at a fixed concentration and

contact time depends on the medium that contaminates the surface. The same product met the 5 log10 criterion for all three bacteria on TSA, BHI and NA, but fell about 1.6 log10 short against the Gram-negative organisms on MacConkey agar. The size of the effect (Cohen's d above 4) and the significant interaction term show that it is not a small variation in method but a systematic, organism-specific loss of activity.

The most plausible explanation is interaction between cationic BAC and the anionic bile salts of MacConkey agar (1.5 g/L). Bile salts are amphiphilic anions with detergent properties [4], and anionic-cationic surfactant pairs associate strongly into ion pairs and mixed aggregates of much lower surface activity [5,6]. Sequestration of BAC lowers the free, active concentration at the cell surface. Three observations are consistent with this interpretation: the effect was largest for the organisms with the least margin over their MIC (*P. aeruginosa*, two-fold; *E. coli*, four-fold) and negligible for *S. aureus* (eight-fold); the MIC of BAC for *E. coli* rose ten-fold in the presence of deoxycholate; and a longer contact time offset the loss, as expected if the remaining free BAC kills at a reduced rate [18,19]. The tight MBC/MIC ratios of about two imply that a modest loss of available BAC translates into a steep loss of kill.

The reduced fungal activity on SDA is a smaller effect. SDA is acidic (pH 5.6) and rich in dextrose (40 g/L). Altered surface charge on fungal cell walls and a hydrated, organic-rich surface layer that slows diffusion of the active agent are both plausible, but pH and carbohydrate content vary together between media in this design, so their contributions cannot be separated here. The practical conclusion does not depend on the mechanism: fungal contamination on SDA warrants a longer contact time or a complementary agent. Antifungal evaluation of synthetic compounds against yeast and mould species has been reported previously [27].

The formulation also illustrates the limits of a QAC-alcohol product. It was not sporicidal at use concentration, and the apparent rate depended only moderately on concentration (exponent 0.55), so raising the BAC concentration is an inefficient route to sporicidal activity, in keeping with the intrinsic resistance of bacterial spores [17]. A rotation programme that pairs a non-sporicidal agent such as this one with a sporicidal agent is therefore appropriate [14].

Practical implications. Laboratory disinfection procedures should (i) specify a minimum contact time of 10 minutes where media contamination, especially with bile-salt-containing media, is likely; (ii) include media-contaminated surfaces among the challenges used to qualify a disinfectant; and (iii) include a sporicidal agent in the rotation. Our results indicate that the fix is operational rather than chemical, which is attractive because it requires no reformulation.

Limitations. The mechanism was inferred from the pattern of results and from the supplementary MIC shift, and free BAC was not measured analytically. The SDA effect is confounded by pH and carbohydrate content. Selective and fungal media were tested only with relevant organisms, and ten-minute data are tabulated only for TSA and MacConkey agar, so the conclusion that ten minutes suffices for all media rests on those data. The *P. aeruginosa* margin at ten minutes was thin (0.1 log10), and the ten-minute contact time should be regarded as a minimum. Sporicidal kinetics are indicative, based on three concentrations and five time points. Tests used reference strains rather than facility isolates, and an agar surface is not a model of every porous or soiled surface found in practice.

CONCLUSION

A BAC-IPA disinfectant developed by Plackett-Burman screening was stable for 24 months and bactericidal and fungicidal at use concentration, but its efficacy depended on the culture medium: it complied on TSA, BHI and nutrient agar yet fell short against *E. coli* and *P. aeruginosa* on MacConkey agar, most plausibly through ion-pair formation between BAC and bile salts. A 10-minute contact time restored compliance. Disinfectant validation in pharmaceutical microbiology laboratories should therefore include relevant culture media, and procedures should specify contact time and sporicidal rotation accordingly. Future work should quantify free BAC in the presence of bile salts, separate the pH and carbohydrate contributions on SDA, and extend ten-minute testing to all media and to facility isolates. Computational approaches such as QSAR and molecular docking [27,28], and

nanomaterial-based carriers such as carbon nanotubes [29], may also help to model BAC-anion interactions and to develop supplementary or sporicidal formulations.

Acknowledgements

The authors thank the University Institute of Pharmacy, Oriental University, Indore, for providing facilities for this work.

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HOW TO CITE: Saurabh Soni, Sudha Vengurlekar, Dhawal Dorwal, Culture-Medium Composition Modulates the Efficacy of a Benzalkonium Chloride–Isopropyl Alcohol Disinfectant: Development, Evaluation and Contact-Time Remediation for Pharmaceutical Microbiology Laboratories, Int. J. of Pharm. Sci., 2026, Vol 4, Issue 11, 5611-5617. https://doi.org/10.5281/zenodo.24581949