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Development of a Multidose Formulation for a Humanized Monoclonal Antibody Using Experimental Design Techniques

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ABSTRACT

The purpose of this study was to identify optimal preservatives for a multidose formulation of a humanized monoclonal antibody using experimental design techniques. The effect of antimicrobial parenteral preservatives (benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol) on protein stability was assessed using size-exclusion chromatography, differential scanning calorimetry, right-angle light scattering, UV spectroscopy, and potency testing using a cell-based fluorescence-activated cell sorting method. A quick, cost-effective preservative screening test was designed. Combinations of preservatives were examined using an I-optimal experimental design. The protein was most stable in the presence of methylparaben and propylparaben, and was compatible with benzyl alcohol and chlorobutanol at low concentrations. Phenol and m-cresol were not compatible with the protein. The I-optimal experimental design indicated that as an individual preservative, benzyl alcohol was promising. The model also indicated several effective combinations of preservatives that satisfied the antimicrobial efficacy and physical stability constraints. The preservative screening test and the experimental design approach were effective in identifying optimal concentrations of antimicrobial preservatives for a multidose protein formulation; (1) benzyl alcohol, and (2) the combination of methylparaben and chlorobutanol were screened as potential candidates to satisfy the regulatory requirements of various preservative efficacy tests.

KEYWORDS: multidose formulation, preservative, experimental design, monoclonal antibody, protein

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INTRODUCTION

Multidose formulations must contain antimicrobial agents to protect them from microbial contamination during multiple dosage withdrawals from vials. Biopharmaceuticals are often formulated as multidose products because they are expensive and they tend to support the growth of microorganisms. Multidose products are convenient for patient administration, minimize sample wastage when dosage requirements are not known, and can provide dosage flexibility for future drug indications.

Over the past decade, there has been a significant increase in the number of commercial protein products, but few have been marketed in a multidose configuration.[1] The literature on the development of multidose formulations for proteins is also not very extensive. The paucity of multidose protein products may be related in part to the difficulty of selecting appropriate preservatives.

Selection of the optimal preservative(s) is dependent on a number of factors.[1-3] Preservatives need to be compatible with the route of administration and be effective against various strains of fungi and bacteria.[2,3] Preservative activity is pH specific (eg, benzyl alcohol is effective only in the pH range of 4-7), and thus the pH of the formulation limits the use of a number of preservatives. Other formulation components impose additional restrictions; for example, nonionic surfactants such as the Tweens inactivate parabens and phenolic preservatives.[3] Poor aqueous solubility and concentration loss due to adsorption by rubber stoppers are other concerns in ensuring the long-term antimicrobial efficacy of preservatives.[4-6] Acceptance of preservatives in target markets is also important. Many preservatives approved for parenteral use in the United States are not approved in Europe and Japan.[2] Antimicrobial preservatives are also quite toxic, and thus the target population's sensitivity to them needs to be carefully evaluated. For example, benzalkonium chloride and ethylenediaminetetraacetic acid (EDTA) from nebulized solutions have been reported to induce dose-related bronchoconstriction in asthmatics.[7]

The effect of the preservative on protein stability is a major concern. Antimicrobial preservatives are known to interact

with proteins and cause stability problems such as aggregation.[8-10] Thus, identifying formulation-compatible preservatives at concentrations that also provide the desired antimicrobial efficacy can be challenging. In addition, regulatory requirements assert that the antimicrobial efficacy of the formulation must satisfy the preservative efficacy test (PET) requirements of the target markets. The PET requirements of the United States Pharmacopoeia (USP) and the European Pharmacopoeia (EP) differ considerably, imposing additional constraints in developing multidose formulations.[2]

In this study, 6 preservatives were evaluated for compatibility with the antibody formulation (10 mg/mL protein in a histidine buffer, pH 6.0, containing Tween 80 and NaCl) and for antimicrobial efficacy. The preservatives' efficacy against various microbes was screened using a modified USP/EP PET to reduce cost and experiment time. After a preliminary screening of preservatives, an I-optimal experimental design approach was taken to identify the optimum preservative concentrations. The approach reported here might be useful for scientists developing multidose formulations for other biological products.

MATERIALS AND METHODS

Materials

The humanized monoclonal antibody was produced at Protein Design Labs, Inc (Fremont, CA). This study was conducted with 10 mg/mL protein, formulated in histidine buffer at pH 6.0, with Tween 80 and NaCl.

The preservatives benzyl alcohol, m-cresol, and phenol were obtained from Sigma (St Louis, MO) and chlorobutanol, methylparaben, and propylparaben were obtained from USPC Inc. (Rockville, MD).

Methods

Effects on Protein Stability

The compatibility of 6 parenteral preservatives (benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol) with the formulated humanized monoclonal antibody was tested. The preservatives were added to the formulated antibody based on their commonly used concentration ranges in marketed multidose products.[3] Because protein aggregation was suspected as being the primary degradation pathway, the preservatives were initially evaluated by differential scanning calorimetry (DSC), and after 2 days of incubation at 50°C, by visual inspection for appearance and by SEC for soluble aggregates.

Because of the results seen in the preliminary evaluation, additional analyses were done using lower concentrations of the preservatives. Samples were incubated at 5°C and 45°C for 1 week, then analyzed with SEC, fluorescence spectroscopy, UV spectroscopy, and potency testing with a fluorescence-activated cell sorting (FACS) binding assay.

Size Exclusion Chromatography (SEC)

The monomer content, soluble aggregates, and clips due to hydrolysis were monitored by SEC. The analytical system employed consisted of an HPLC (high performance liquid chromatography) pump (Perkin Elmer, Series 410, Shelton, Connecticut) and an autosampler (Perkin Elmer, ISS 2000) connected to a diode array detector (Perkin Elmer, 235C). Two SEC columns (Tosohaas TSK-Gel, G3000SW$_{XL}$) were connected and used for sample separation. The composition of the mobile phase was 200 mM KPO_4, 150 mM NaCl, pH 6.9. Samples were diluted to 1 mg/mL, and a sample volume of 40 µL was injected for analysis. The flow rate was 1.0 mL/min, and detection was at 220 and 280 nm.

Fluorescence Spectroscopy

Fluorescence spectroscopy was used to monitor changes in the protein tertiary structure. Measurements were done on the Luminescence Spectrometer, LS50B, from Perkin Elmer (E5087). Samples were diluted 100-fold and excited at 280 nm. The emission spectrum was monitored from 285 to 425 nm at a scan speed of 150 nm/min.

DSC

A decrease in the denaturation temperature reflects a destabilizing effect of the preservative on formulation stability. The denaturation temperature (T_m) of the sample was measured using the Pyris 1 differential scanning calorimeter (Perkin Elmer). A sample volume of 50 µL was taken from a 10 mg/mL protein sample and sealed in a stainless steel pan. The sample was held at 32°C for 2 minutes and heated to 100°C at the rate of 10°C/min.

Potency Testing

The biological activity (potency) of the protein was measured using a cell-based FACS method, based on the

Table 1. USP 24 and EP 2 Requirements for Preservative Efficacy Testing*

Time Point	USP 24 Requirements	EP 2 Requirements	
		Suggested (A Criteria)	Minimum (B Criteria)
Requirements for bacterial log reduction			
6 hours	Not required	3	Not required
24 hours	Not required	No recovery	1
2 days	Not required	No recovery	Not required
7 days	1	No increase	3
14 days	3	No recovery	Not required
21 days	No increase	No recovery	Not required
28 days	No increase	No recovery	No increase
Requirements for fungal log reduction			
7 days	Not required	2	Not required
14 days	No increase	No increase	1
28 days	No increase	No increase	No increase

*USP indicates United States Pharmacopoeia; EP, European Pharmacopoeia.

binding of the antibody antigen expressed on human T cells.

Preservative Screening Test (Bactericidal/Fungicidal Activity)

The efficacy of the preservative against various microorganisms was measured using a modified USP/EP PET (referred to as preservative screening test in this document). Tests were conducted at Microconsult Inc (Dallas, TX). In the procedure, formulations were tested against the following microorganisms: Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, and Aspergillus niger. The 3 bacterial strains were inoculated together at a total concentration of ~10^5 cfu/mL, as were the 2 fungi. Samples were incubated for 7 days at room temperature (25°C), and the total bacterial and fungal counts were measured using a colony counter. The log reduction (LR) values for the bacterial and fungal counts were calculated as log (initial count/final count).

In the unmodified USP/EP PETs, each microorganism is tested separately at a concentration of ~10^5 cfu/mL. The USP and EP regulatory guidelines[2] are listed in **Table 1**; note that the EP guidelines are more stringent than those of the USP and that the EP guidelines offer a minimal level that must be achieved (B criteria) and a suggested level that is recommended (A criteria). The PET was modified in this study to reduce the total sample requirement and cost per analysis. Although the bacterial and fungal strains were not tested individually at specified concentrations, by comparing the overall bacterial and fungal LR values with the regulatory requirements, one can assess the efficacy of the preservative against these microorganisms.

I-Optimal Experimental Design

An I-optimal experimental design was used to evaluate and model the effects of single and combined preservatives on formulation stability and antimicrobial efficacy. We were especially interested in evaluating whether combinations of preservatives enhanced the antimicrobial efficacy of the formulation, because the preservatives were compatible with the formulation at only low concentrations. The preservatives benzyl alcohol, chlorobutanol, methylparaben, and propylparaben were examined

Table 2. I-Optimal Experimental Design: Comparison of Measured and Predicted Log Reduction Values for Bacteria and Fungi*

					Log Reduction Values			
	Preservative Concentration, %				Bacteria		Fungi	
SN†	BA	CB	MP	PP	Measured	Predicted‡	Measured	Predicted§
1 (2)	0.33	0.10	0.05	0.005	2.11	2.90 ± 0.10	3.72	3.69
2	0.00	0.20	0.03	0.000	2.36	2.32 ± 0.19	1.75	1.75
3	0.00	0.05	0.10	0.000	2.59	2.58 ± 0.19	3.72	3.72
4	0.75	0.10	0.05	0.005	3.83	3.40 ± 0.14	3.72	3.80
5	0.47	0.20	0.05	0.010	3.83	3.67 ± 0.18	3.72	3.75
6	0.47	0.10	0.00	0.000	3.83	3.67 ± 0.10	3.72	3.75
7	0.66	0.20	0.10	0.000	3.83	3.84 ± 0.19	3.24	3.23
8	0.00	0.00	0.07	0.010	1.54	1.53 ± 0.19	3.72	3.72
9	0.33	0.10	0.05	0.005	3.83	2.90 ± 0.10	3.72	3.70
10	0.00	0.15	0.00	0.010	0	–0.01 ± 0.19	2.72	2.72
11	0.47	0.00	0.10	0.005	3.83	3.67 ± 0.18	3.72	3.75
12 (3)	0.66	0.00	0.00	0.010	3.83	3.83 ± 0.11	3.72	3.72
13	0.75	0.20	0.00	0.005	3.49	3.52 ± 0.18	3.72	3.69
14	0.00	0.20	0.10	0.007	3.83	3.81 ± 0.19	3.72	3.72
15 (2)	0.75	0.00	0.05	0.000	3.83	3.91 ± 0.13	3.72	3.70
16 (2)	0.00	0.00	0.00	0.003	0	–0.01 ± 0.13	0.00	0.00
17	0.75	0.10	0.10	0.010	3.83	4.00 ± 0.18	3.72	3.69

*SN indicates sample number; BA, benzyl alcohol; CB, chlorobutanol; MP, methylparaben; PP, propylparaben.
†Numbers in parentheses indicate replicates included in the design. In those cases, mean values are given.
‡Response variation is reported based on 95% confidence limits.
§Pooled standard deviation for this data set was 0.

in the concentration ranges of 0 to 0.75%, 0 to 0.2%, 0 to 0.1%, and 0 to 0.01%, respectively.

The formulations were prepared by adding the preservatives at the desired concentrations as per the I-optimal design table (**Table 2**), generated using the software Strategy (Experiment Strategies Foundation & Process Builder, Inc., Bremerton, WA). All samples were incubated at 37°C for 9 weeks. Because protein aggregation was known to be the primary degradation pathway, protein stability was examined by SEC and right-angle light scattering to monitor the formation of soluble and insoluble aggregates, respectively, and by UV spectroscopy to monitor changes in the protein concentration. The biological activity of the samples was assessed after 1 month at 37°C. Furthermore, to assess the antimicrobial efficacy of the formulations, samples were examined by the preservative screening test at the initial time point. Samples were incubated at room temperature, and the aerobic plate counts were measured based on the minimum requirements of the USP and EP PETs at 24 hours, 7 days, and 14 days.

In the I-optimal model, the main effect and the interaction effects of various factors are determined by fitting the data to a second-order quadratic equation:

$$Y = b_o + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i<j} \sum b_{ij} x_i x_j \qquad (1)$$

where Y is the dependent variable or the measured response and x_i represents the independent variable that corresponds to the concentration of excipient i. The

Table 3. Preliminary Screening of Preservatives to Evaluate Compatibility With the Formulated Protein*

Preservative	Concentration, %	Visual Analysis	Percent Monomer (by SEC)	T_m, °C (by DSC)
Benzyl alcohol	2	Precipitated	ND	72.3
	1.0	Slightly cloudy	13.4	75.8
	0.5	Clear	92.3	78.5
Chlorobutanol	0.5	Clear	3.3	78.5
	0.2	Clear	ND	79.9
	0.1	Clear	96.9	ND
Methylparaben	0.1	Clear	91.3	ND
	0.05	Clear	ND	80.4
Propylparaben	0.02	Clear	85.3	80.6
Phenol	0.5	Cloudy	20.0	ND
	0.1	Slightly cloudy	62.7	ND
m-cresol	0.3	Precipitated	ND	ND
	0.1	Precipitated	ND	ND
Control	0.0	Clear	97.4	80.1

*SEC and visual analysis were done after 2 days of incubation at 50°C. DSC analysis was conducted at the initial time point, t_0. SEC indicates size-exclusion chromatography; DSC, differential scanning calorimetry; ND, not determined.

model coefficients determined by regression analysis define the response surface; b_O is a constant term, b_i indicates the main effect of excipient x_i, and b_{ij} represents the interaction effect between excipients i and j. These model coefficients were used to generate response surfaces that simulate the effect of preservatives on the desired response.

RESULTS

Effects on Protein Stability

The results from the preliminary testing after 2 days of incubation at 50°C indicated that protein stability correlated with preservative concentration (**Table 3**). Benzyl alcohol caused sample precipitation at concentrations ≥2%. At a concentration of 1.0%, the sample was slightly cloudy, and there was a significant loss in the monomer content due to the formation of soluble aggregates (monomer content was only ~13%). However, at 0.5% benzyl alcohol, the sample was clear and the monomer content was ~92%. Previously, the destabilizing effect of benzyl alcohol on recombinant human interferon gamma has been reported to be due to the disruption of the protein's tertiary structure, making the protein more susceptible to aggregation.[10] In the presence of chlorobutanol, the formulations were clear; however, as with benzyl alcohol, the protein however, as with benzyl alcohol, the protein formed soluble aggregates at higher preservative concentrations. The monomer content values in samples containing 0.5% and 0.1% chlorobutanol were ~3% and 97%, respectively. The protein was more stable in the presence of methyl- and propylparaben. Despite being tested at their highest recommended concentrations (0.1% for methylparaben and 0.02% for propylparaben), the monomer content values were ~91% and 85%, respectively. When tested under identical conditions, the control formulation containing no preservatives showed ~97% monomer. Phenol and m-cresol considerably destabilized the protein; phenol caused the formation of both soluble and insoluble aggregates, while m-cresol precipitated the protein. Thus, these 2 preservatives were not evaluated further in this study.

The denaturation temperature of the control formulation was ~80°C. In the presence of benzyl alcohol, the denaturation temperature was considerably reduced; for formulations containing 2.0% and 0.5% benzyl alcohol, the measured T_m values were 72.3 and 78.5°C, respectively. Similarly, at 0.5% chlorobutanol, the T_m dropped by 1.6°C relative to the control formulation. The addition of methyl- and propylparaben, however, did not affect the denaturation temperature. These results are thus consistent with the SEC results, indicating the relatively greater compatibility of the parabens with the formulated protein

Table 4. Effect of Preservatives on Protein Stability and Antimicrobial Efficacy*

Preservative	Concentration(%)	Monomer (%), 45°C	Potency (%), 45°C	Bacteria x 10^4 (cfu/mL), † 25°C	Fungi x 10^5 (cfu/mL), ‡ 25°C
Benzyl alcohol	0.75	87.9	74	0	0
	0.5	93.9	75	0	0
	0.1	97.7	81	TNTC	12
Chlorobutanol	0.2	96.7	63	46	0
	0.1	97.5	67	TNTC	14
	0.05	97.6	60	TNTC	18
Methylparaben	0.1	97.2	75	56	0
	0.05	97.4	75	TNTC	2
	0.01	98.3	73	TNTC	26
Propylparaben	0.01	97.3	68	TNTC	0
	0.0075	97.9	74	TNTC	2
Control	—	97.5	69	TNTC	20

*Samples were incubated for 1 week at the indicated storage temperatures. TNTC indicates too numerous to count.
†Total bacterial count = 8.78 x 10^4 cfu/mL.
‡Total fungal count = 2.84 x 10^5 cfu/mL.

and the instability of the protein at higher concentrations of benzyl alcohol and chlorobutanol.

Based on the results obtained using preservatives at typical commercial concentrations in the preliminary investigation, preservative concentrations were reduced in further analyses (**Table 4**). SEC results indicated that at these lower concentrations, the monomer content for all samples was >98% at 5°C (data not shown). After 1 week at 45°C, the monomer content in samples containing chlorobutanol, methylparaben, and propylparaben was comparable to the control formulation (~97%); however, samples formulated with benzyl alcohol were unstable because of the formation of soluble aggregates (**Table 4**). At its highest concentration (0.75%), the loss in monomer content was ~9.5% relative to the control formulation. To correlate the observed aggregation with changes in the protein tertiary structure, all samples were analyzed by fluorescence and UV spectroscopy. However, no changes in the tertiary structure were apparent, possibly because the extent of aggregation in the samples was not sufficient to cause detectable changes in the tertiary structure (data not shown). Previously, changes in the protein tertiary structure in the presence of benzyl alcohol have been reported based on circular dichroism spectroscopy measurements at concentrations ≥0.9%.[10]

Potency was measured only in samples incubated at 45°C. Because of the inherent variability of this assay (SD ~8%), the potency of the preservative-containing formulations was equivalent to that of the control formulation containing no preservatives (based on 95% confidence limits). Our earlier studies have shown that at high temperatures, structural changes in the protein due to deamidation and oxidation affect its biological activity. However, at ambient temperature, these processes slow down considerably and the molecule satisfactorily retains its biological activity for the target shelf life. Thus, the data reported here indicate that under the examined conditions, the loss in biological activity at 45°C is not catalyzed by the preservatives in the formulation.

Preservative Screening Test

Results of the preservative screening tests showed that the formulations containing 0.75% and 0.5% benzyl alcohol are potential candidates to meet the USP/EP criteria (**Table 4**). Both formulations demonstrated a complete kill of the tested bacterial and fungal species after 7 days. For all other samples, either the total bacterial count after 7 days was too numerous to count, or no effective reduction in the bacterial count was observed. The antimicrobial efficacy was also satisfactory against fungi for formulations containing at least 0.5% benzyl alcohol, and for formulations containing parabens and chlorobutanol at their highest concentration. However, in the stability testing reported above, the stability of the protein strongly correlated with the concentration of benzyl alco-

hol. It is thus important to further examine whether combining preservatives at lower concentrations can lead to the desired antimicrobial efficacy without affecting protein stability.

I-Optimal Analyses

The SEC, right-angle light scattering, and UV spectroscopy responses after 9 weeks of incubation at 37°C and the bioactivity response after 1 month at 37°C were modeled using the I-optimal design (data not shown). The regression results for the responses were not statistically significant, and the samples could not be statistically distinguished from the control formulation. Thus, preservatives in the examined concentration range did not adversely affect the stability of the samples. However, the formulations differed markedly in their antimicrobial efficacy in the preservative screening test (**Table 2**).

The reduction in the bacterial and fungal counts following 14 days of incubation at room temperature was taken as the measured response and modeled using the I-optimal design. The data at the 24-hour and 7-day time points also followed a similar trend (data not shown). The regression yielded a set of coefficients that correlates the concentration of the preservatives to the LR in the bacterial and fungal counts (see Equation 1). Thus, the response in the region of interest can be simulated to optimize the formulation components. Excellent agreement was observed between the experimentally measured and model-predicted responses (**Table 2**), confirming a good fit between the model and the experimental data.

The b-coefficients determined by regression analysis are listed in **Table 5**. Benzyl alcohol, chlorobutanol, and methylparaben showed statistically significant antimicrobial efficacy against bacteria, the effect being strongest for benzyl alcohol, followed by methylparaben and chlorobutanol. Propylparaben, on the other hand, was not effective against the tested bacterial strains. Interaction effects were also statistically significant between various preservatives; the strongest positive interaction (synergistic effect) was between methylparaben and propylparaben, and the strongest negative interaction was between benzyl alcohol and methylparaben. Other positive interaction effects included benzyl alcohol and propylparaben, and chlorobutanol and methylparaben. The b-coefficients for fungi were also statistically significant. All selected preservatives had a positive antifungal efficacy, the strongest effect being observed for benzyl alcohol, followed by methylparaben. All interaction effects were, however, negative.

The efficacy of the single and combined preservatives was evaluated by comparing the LR values predicted by the model with the regulatory requirements. **Figures 1A** and **1B** show the effects of benzyl alcohol and chlorobutanol on the LR of the bacterial and fungal counts, respectively. The antimicrobial efficacy against bacteria and fungi increased with increasing concentrations of benzyl alcohol and chlorobutanol, but it is unlikely that chlorobutanol alone can provide adequate protection against bacteria or fungi. The simulations predict that as single preservatives, 0.75% benzyl alcohol and 0.2% chlorobutanol (their maximal concentrations) would provide LR values of 4.8 and 2.0, respectively for bacteria, and of 3.7 and 1.2, respectively, for fungi. These results indicate that benzyl alcohol is likely to be effective in preserving the formulation against both bacteria and fungi; however, in the examined concentration range, chlorobutanol may not meet the regulatory requirements.

The results also show that combinations of benzyl alcohol and chlorobutanol do not enhance the antimicrobial efficacy against bacteria; however, they can enhance the antimicrobial efficacy against fungi. For example, by using 0.75% benzyl alcohol and 0.125% chlorobutanol, the LR in the fungal count can be increased from 3.7 to 4.6. However, the bacterial LR under these conditions drops from 4.8 to 4.3. These model predictions can also be advantageous in seeking alternatives if protein stability, preservative toxicity, or other factors require the preservative to be in a specific concentration range.

Combining chlorobutanol and methylparaben has a synergistic effect on antimicrobial activity against bacteria and fungi (**Figures 1C** and **1D**, respectively). The model simulations indicated maximal LRs of 2.0 and 2.3 for the individual preservatives against bacteria; their combination resulted in a significant improvement of up to 4.0 LRs. The LR in the fungal count increased marginally from 3.2 to 3.9. Thus, the combination of chlorobutanol and methylparaben may offer a promising alternative to the use of benzyl alcohol.

Based on these results, to evaluate the efficacy of the preservative screening approach undertaken in this study, the protein was formulated with 0.75% benzyl alcohol, and its stability and bioactivity were monitored over time (data not shown). The antimicrobial efficacy of the preservative was monitored by the USP and EP PETs. Results indicated that in samples containing 0.75% benzyl alcohol, protein stability was comparable to that of the control formulation and the USP and EP (criterion B only) regulatory requirements were satisfied.

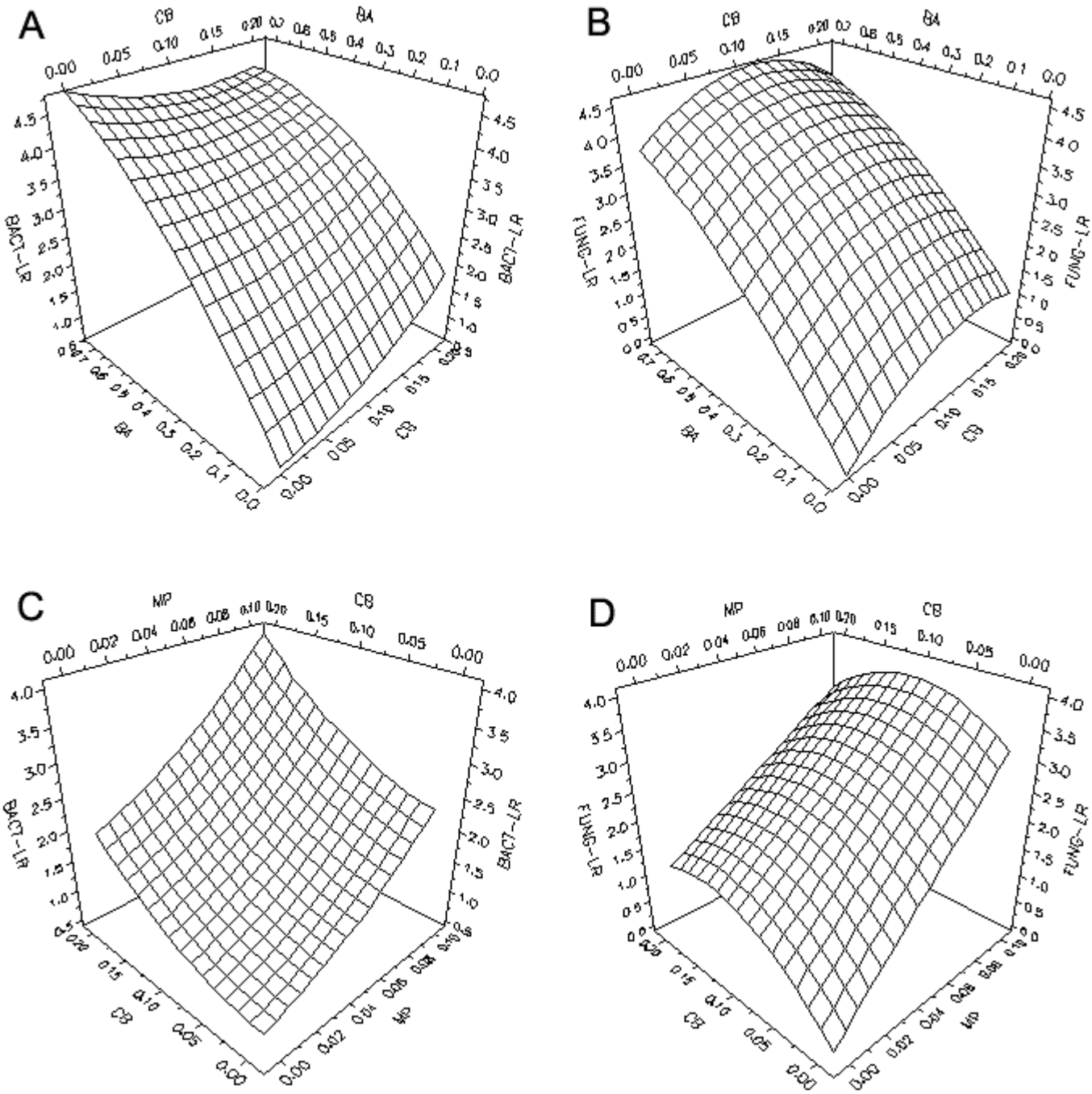

Figure 1. Response surface plots indicating the bacterial and fungal count as a function of the concentration of 2 preservatives. Counts were measured after 14 days of incubation at room temperature: effect of benzyl alcohol and chlorobutanol (A) on the bacterial count and (B) on the fungal count; effect of chlorobutanol and methylparaben (C) on the bacterial count and (D) on the fungal count.

CONCLUSION

The effects of 6 preservatives (benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol) on the stability of a humanized monoclonal antibody were examined in order to develop a multidose intravenous formulation. Preservatives were screened based on their effect on the physical stability of the formulations using various analytical techniques and on their antimicrobial efficacy using a modified PET. Protein stability in the presence of the parabens and low concentrations of chlorobutanol compared well with that

of the control formulation. Benzyl alcohol caused significant aggregation at high concentrations (≥1.0%); however, it was the most effective preservative in maintaining antimicrobial efficacy against bacteria and fungi. Phenol and m-cresol were not compatible with the protein and caused protein precipitation.

An I-optimal experimental design was used to monitor the individual effects of each preservative and to examine combinations of preservatives that result in a synergistic effect. Using these results, we conclude that as a single preservative, only benzyl alcohol has the potential to meet the regulatory requirements. As combinations, benzyl alcohol-chlorobutanol and benzyl alcohol-methylparaben enhanced the antimicrobial efficacy of the formulation against fungi, and chlorobutanol and methylparaben enhanced the antimicrobial efficacy against both bacteria and fungi, at all concentrations of both preservatives. Thus, the use of modified PETs to assess the antimicrobial efficacy of various preservatives and the I-optimal experimental design approach employed in this study proved to be effective in testing the feasibility of developing multidose formulations for humanized monoclonal antibodies.

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