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Preservative Efficacy Screening of Pharmaceutical Formulations Using ATP Bioluminescence

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The preservative challenge test is a method used to determine the efficacy of a preservation system in a pharmaceutical or cosmetic formulation. However, such testing is a labor-intensive, repetitive task often requiring days before results can be generated. Several alternatives to traditional colony-count techniques have been developed. A study using pure suspensions of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Candida albicans,* and *Aspergillus niger* showed that the accuracy, repeatability, and linearity of the Pallchek™ luminometer ATP bioluminescence (ATP-B) system was equivalent to the traditional colony-count method. In any case, the method proved sensitive enough to follow the effect of preservatives on a number of test microorganisms, indicating the applicability of the ATP-B method for preservative screening studies in various pharmaceutical formulations.

Keywords pharmaceutical product development studies; preservative efficacy test; screening test; ATP bioluminescence; rapid microbiology

INTRODUCTION

Antimicrobial preservatives are used to prevent or inhibit the growth of microorganisms that could present a risk of infection or degradation of the medicinal product (Beveridge, 1999). The list of possible materials for a company to choose from if they wish to use an established antimicrobial preservative in a new product is quite limited. The costs associated with the introduction of a novel excipient can be extremely high because the substance will, in effect, be treated as a new active ingredient in terms of the data requirements to support its use (Matthews, 2003). Although the chemical, physical, and microbiological properties of a preservative are clearly important, they do not provide sufficient information to predict that a formulated product will be adequately preserved (Hugo & Russel, 1999). Several factors influence the activity of a preservative (Beveridge, 1999). These include its concentration, pH, temperature, chemical composition, and condition of microorganisms, the presence or absence of interfering substances, and the possible interactions of a preservative with containers and closures. These aspects must be considered when choosing a proper antimicrobial agent, as a preservative, as an integral part of the early development of a pharmaceutical formulation (Russel, 2003).

Consequently, it is difficult to predict accurately the ultimate effectiveness of a preservative in any but the simplest pharmaceutical formulation. It is thus necessary to obtain some assurance of its likely in-use performance in a formulated product. A challenge test (also known as a preservative effectiveness test [PET] or antimicrobial effectiveness test) is a procedure to determine whether a formulated cosmetic, pharmaceutical, or other type of product is adequately preserved to prevent proliferation from raw products and during consumer use (Hodges & Denyer, 1996). Different pharmaocopeias, for instance European Pharmacopoeia (EP) (Anonymous, 2002), British Pharmacopoeia (BP) (Anonymous, 2000), and United States Pharmacopoeia (USP) (Anonymous, 2003), contain methods that are intended for the assessment of the efficacy of an antimicrobial preservative in pharmaceutical products. These methods vary only slightly from one pharmacopoeia to another and basically share the following experimental properties (Fels, 1995). The product to be tested is inoculated with five different strains of microorganisms (bacteria and fungi). The contaminated product is sampled after different, well-defined time intervals and the number of viable organisms are determined. The preservative must cause a specified reduction in viable count, which must be maintained over a period of 28 days

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(Connolly, Bloomfield, & Denyer, 1994; Fels, 1995). The current method is not economical in labor and material. It relies on the growth of microorganisms; the results take days or, as in the case of PET, months. Many companies are manufacturing products under increasing pressures of time, and the delays in product release can have significant cost implications due to excessive in-process retention of inventory. Moreover, the traditional microbiological techniques require skilled personnel for sample handling and result interpretation (Morris, 1998).

New methods of rapid microbial detection are emerging in modern microbiology (Wills et al., 1995). In this area, the microcolony fluorescence staining method, enzyme-linked immunoassay, and polymerase chain reaction (PCR) method have been studied over the past 15 years (Takahashi, Nakakita, Watari, & Shinotsuka, 2000). Major advantages claimed for these methods are that they are more economic in use of materials requiring shorter time periods than typical colony-count methods to obtain final results. A considerable amount of data are available concerning rapid methods of detecting microbial viability in food microbiology. In contrast, introduction of utilization in the pharmaceutical industry has been slow, probably because of their lack of sensitivity (Connolly, Bloomfield, & Denyer, 1993). One obvious application of rapid methods in the pharmaceutical industry (and also cosmetic and toiletry industries) is for PET. In a previous study (Connolly et al., 1993), three rapid microbiological methods, impedance, the direct epifluorescence microcolony method (DEFT-MEM), and adenosine triphosphate bioluminescence (ATP-B) assay, were evaluated for their applicability to PET. It was concluded that impedance represents a valid method for PET. Contrarily, neither DEFT-MEM nor ATP-B at its current level of method development was considered as satisfactory. Over the past 10 years or so, a great deal of effort has been put to make use of bioluminescence process like that with the American firefly *Photinus pyralis*. Applications are based on capturing the microorganisms, releasing the ATP from the cell, and measuring the amount of bioluminescence generated. A high reading of relative light units (RLUs) indicates that the sample contains a high number of microorganisms, provided the background ATP level is low (Lundin, 1989). Light is produced within seconds and can be measured with a luminometer of which there are several types. In the mean time, more effective extractants, reagents, and more sensitive luminometers have been developed. Based on this technology improvement, confirmation of the applicability of the ATP-B method at its current level of development for preservative efficacy screening of pharmaceutical formulations was our primary intention. The comparison of the rapid ATP-B method with current culture-based method and its validation following guidelines of the Parenteral Drug Association (PDA) Technical Report No. 33 "Evaluation, Validation, and Implementation of New Microbiological Testing Methods" were performed. In our study, we used the Pallchek™ luminometer (Pall Life Sciences, Portsmouth, Hampshire, UK), based on ATP-B method, which has been shown to be more sensitive with a limit of detection down to 100 cells/mL. Therefore, it could be a useful tool to provide a rough estimation of microbial levels concerning various aspects of pharmaceutical manufacturing including raw material, in-process, and finished product testing.

A number of considerations must be taken into account when using the ATP-B method, including sample effects, efficiency of ATP extraction, and sensitivity of the reagent. The effect of the sample itself on the bioluminescence assay is sometimes unpredictable. Occasionally, products contain detergents and salts that could impact the bioluminescence reaction (Simpson & Hammond, 1991). Some sample ingredients may be derived from natural sources and can contain high levels of nonmicrobial ATP. High background ATP levels can give false-positive results. Therefore, the occurrence of false positives has to be reduced, still keeping sufficient sensitivity for detecting the microorganisms (Takahashi, Nakakita, Watari, & Shinotsuka, 2000). The detection of microorganisms using luciferin–luciferase reagents requires an extractant to lyse the cells and release ATP into solution. The extractant must be sufficiently aggressive to attack a broad range of microbes, it must not affect the activity of the luciferase enzyme, and it should exibit some chaotropic action to inactivate intracellular enzymes, which would use free ATP to begin repairing the bacterial structure (Stanley, 1989). The producer has also to ascertain the sensitivity and stability of bioluminescence reagents (Lundin, 1989). All these aspects were taken into account in our study as well in an attempt to prove the applicability of the ATP-B method for preservative efficacy screening of pharmaceutical formulations.

MATERIALS AND METHODS

Media and Solutions

The following culture media, solutions, and reagents were used: Tryptone Soya Agar (TSA; Merck, Darmstadt, Germany, 1.05458), Sabouraud Dextrose Agar (SDA; Merck 1.05438) with chloramphenicol 0.05 g/L (Calbiochem, San Diego, California, USA), Ringer solution (Merck 1.15525), phosphate-buffered saline (PBS; Oxoid, Cambridge, UK, BR 14a). Neutralized peptone water (NPW) contained (g/L) in distilled water the following: peptone (Merck 1.07214), 1.0; sodium chloride, 8.9; lecithin (ICN, Pharmaceuticals Inc., Aurora, Ohio, USA, 151539), 3.0; Tween 80 (Merck 8.22187), 30.0. For ATP-B assay, we used Pall HS kit (7142) with extractant and bioluminescent reagent.

Microorganisms

In accordance with the pharmacopoeias, the following strains of microorganisms were used: *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), and *Aspergillus niger* (ATCC 16404).

Bacteria were grown overnight at 37°C on TSA, whereas the two fungi were cultured on SDA at 25°C for 3–5 days.

Preparation of Samples for Validation

For validation studies, microorganisms were harvested into a Ringer solution by gentle agitation and diluted to contain the required number of colony-forming units (cfu) per mL, approximately 10^8 cfu/mL. The Ringer solution for harvesting *A. niger* contained 0.05% (wt/vol) of Tween 80. Tenfold dilutions were performed with the Ringer solution to obtain final testing concentrations ranging from 10^7 to 10^2 microorganisms/mL.

Preparation of the Test Formulations

For preservatives studies, varying quantities of stock solutions containing 500 mg/mL methyl parahydroxybenzoate (MHB; Palco Chemiehandel GMBH, Kolbermoor, Germany, 6686604) were added to 20 mL volumes of PBS and 20 g of hydrogel to give a range of concentrations.

Hydroxyethylcellulose gel was prepared from hydroxyethylcellulose, 2.5% (wt/wt) (Natrosol 250), demineralized water and glycerol, 10.0% (wt/wt), according to the procedure (Anonymous, 2005). The preparation was stirred continually until it set. The preservative solution was added prior to setting, and the creams so formulated were left to equilibrate for 24 h at room temperature. The maximum concentration of alcohol incorporated into the test system was 6% (vol/vol), which is considered not to exert any bactericidal effect (Connolly et al., 1994).

For PET a semiliquid topical pharmaceutical preparation (nasal spray) was chosen, which contains budesonide and inactive substances such as disodium versenate, potassium sorbate, anhydrous glucose, microcrystalline cellulose, carboxymethyl cellulose sodium, polysorbate 80, hydrochloric acid, and purified water.

Determination of Colony-Forming Units

We used the pour plate viable counting method to determine cfu. Samples for colony counting were diluted as required in NPW, and 1 mL of the sample in duplicate was plated on TSA. Fungal plate counts were performed on SDA. Colony counts were determined after incubation according to EP-prescribed ranges at 30°C for 48 h for bacteria and at 25°C for 72 h for fungi.

ATP-B Determinations

ATP experiments were performed on the Pallchek™ luminometer (Pall Life Sciences). The bioluminescence reagent kit used for the analysis was the Pall Bioluminescence High Sensitivity Reagent Kit (Part No. 7,142, Pall Life Sciences). Freeze-dried bioluminescence reagent, extractant, and reconstitution buffer are proprietary solutions, provided by Pall in their kits. Bioluminescence reagent consists of purified, freeze-dried luciferin–luciferase, which is rehydrated in reconstitution buffer, containing buffer, salts, and other components. We used aseptic technique during reconstitution and dispensing of reagents to prevent ATP contamination. Furthermore, the aluminum test plate was wiped with alcohol at the end of each day and rinsed with sterile, ATP-free, deionized water prior to testing to minimize background reading. ATP standard solution (adenosine 5′-triphosphate, 400 ng/mL, 7.25×10^{-7} M) was also from Pall Life Sciences and included in Pall High Sensitivity ATP Correlation Kit (Part No. 7,150, Pall Life Sciences).

ATP Assay

Before testing, we reconstituted the bioluminescence reagent with a reconstitution buffer in the amber glass bottle. For testing, we placed a Multipurpose Sample Holder (Pall Life Sciences, Part no. 7,146) in the recess of the aluminum test plate and added 100 µL of sample dilution to the holder and then applied 100 µL of the extractant using a calibrated micropipettor with ATP-free tips. After 15 s we applied 100 µL of reconstituted bioluminescence reagent and placed the Pallchek luminometer on the aluminum test plate and took a reading of light output (RLUs) within a 5 s.

Standardization Procedure

A reduction in light output due to possible sample interference using standardization procedure was investigated. Therefore, the light emitted from the sample after addition of ATP standard was compared with the light emitted from an equal concentration of ATP standard in demineralized water.

We prepared a 10-fold serial dilutions of ATP standard solution (adenosine 5′-triphosphate, 400 ng/mL, 7.25×10^{-7} M) in demineralized water and preserved sample (PBS, hydrogel). We continued serial dilutions until ATP was diluted to 1×10^{-12} M. For each ATP dilution in turn, we transferred 100 µL of the ATP dilution to a sample holder and added 100 µL of bioluminescence reagent. We immediately placed the Pallchek luminometer over the sample and took a reading within 5 s.

Statistical Evaluation

A two-way ANOVA as regards to results obtained by ATP standard dilution in demineralized water or preserved sample at each time interval was performed. The suitability of the method was further evaluated with statistical test of the intercept and slope at $\alpha = .05$.

Validation

To compare the rapid method against the standard plate count method and to determine the suitability of the method for rapid and sensitive routine microbial analysis, we validated the system using several different microorganisms: four bacteria, one mould, and one yeast. We evaluated the following validation parameters: background signal, accuracy, repeatability, linearity, limit of detection, and equivalence.

The background is any Pallchek reading not caused by microbial presence and measured by the Pallchek unit in RLU. Before testing, we determined the background counts by measuring RLU of diluent solution. Final background reading should be between 100 and 150 RLUs according to producer instructions.

The accuracy of the Pallchek luminometer was compared with that of the classical method. PDA defines accuracy of a method as the closeness of the test results obtained by that method to the true value, which is the number of microorganisms added into a Ringer solution. The acceptance criterion is at least 70% recovery.

For repeatability measurements, a suspension of test microorganisms at the upper end of the range was prepared and serially diluted down to lower concentrations. Two suspension concentrations were tested (10^3 and 10^8 cfu/mL), and the relative standard deviation (*RSD*) was calculated. The method is repeatable if *RSD* from 10 repetitions is less than 30%.

For linearity, a suspension of theoretically 10^8 cfu/mL cells was prepared in a Ringer solution. Four successive tenfold dilutions were then performed in Ringer containing theoretically 10^5, 10^4, 10^2, and 10 test microorganisms per mL. The number of microorganisms in each of the five bacterial suspensions was determined five times by the Pallchek luminometer and the determination coefficient (r^2) was calculated. The determination coefficient must be equal or greater than .9000.

The equivalence is a measure of how similar the test results are to those of the method it is intended to replace. The parameter of equivalence was evaluated in repeatability and linearity of the method. For this purpose, serial tenfold dilutions ranging from 10^7 to 10^1 microorganisms per mL were prepared in a Ringer solution from suspensions of the six species. Samples were taken from each of these dilutions, and the number of viable microorganisms was determined by the plate count and the ATP-B method. For each species of microorganism, a two-way ANOVA as regards to results obtained by either method at each time interval was performed. No significant difference should be obtained at the significance level $\alpha = .05$. The suitability of the method is further confirmed with the *t*-test of the intercept and slope at $\alpha = .05$.

Finally, the limit of detection, that is, the lowest number of microorganisms in a sample that can be detected but not necessarily quantified, was investigated. Suspensions of test microorganisms were prepared, that contained approximately the same number of microorganisms as predicted and the number of viable microorganisms was determined by the ATP-B method. The measurement was repeated five times and the *RSD* was calculated. A limit of detection less than 10^3 cfu/mL was expected to prove the usability of the ATP-B method.

Preservative Efficacy Screening Test

For preservative efficacy screening test, three experimental formulations were prepared, that is, PBS, hydrogel, and a semiliquid topical pharmaceutical preparation (nasal spray). All media were inoculated individually with bacterial and fungal test suspensions, that is, *S. aureus*, *P. aeruginosa*, *A. niger*, and *C. albicans* to give an initial viable count of about 10^7 cfu/mL for bacterial strains and *A. niger* to give a count of about 10^6 cfu/mL for fungal strains, respectively. The preparations were well mixed to ensure a homogenous microorganism distribution and incubated at 25°C and protected from light. In case of PBS and hydrogel, 0, 0.3, and 0.6% (wt/wt) concentrations of preservative were added as required to produce log reductions ranging from 0 to 6 logs, when there are no detectable survivors. At 0, 6, 24, 48, and 168 h, 1 g of sample was transferred to 9 mL of NPW. After mixing for 30 s and a 5 min neutralization period, the number of surviving microorganisms was determined by the Pallchek bioluminesence method and efficacy of preservatives statisticaly evaluated using one-way ANOVA.

On a semiliquid topical pharmaceutical preparation, compliance with EP for PET concerning topical formulations was investigated. The number of surviving microorganisms was determined by the ATP-B and the pour-plate count method on TSA or SDA plates, where cfu were counted after 3- and 5-day incubations at 30°C for bacteria and at 25°C for fungi, respectively. All counts were performed in duplicate. A growth control with the medium alone on day 0 was included for each organism and served as an inoculum control, assuring that a consistent and adequate number of viable microorganisms were added to the formulation. A two-way ANOVA as regards to results obtained by either method at each time interval was performed. No significant difference should be obtained at the significance level $\alpha = .05$.

RESULTS AND DISCUSSION

To confirm the applicability of the ATP-B method for preservative efficacy screening of pharmaceutical formulations was our primary intention. The first step toward this goal was validation of the ATP-B method on the simplest liquid system.

Percentage of recovery of concentrated and diluted suspensions of microorganisms was used to demonstrate accuracy. The results in Table 1 show that percentage recovery by the rapid test method is high and in accordance with prescribed criteria, that is, at least 70% recovery. Mean values, calculated for the six organisms tested, are 115.7% recovery for concentrated suspension and 102.4% recovery for diluted suspension.

For repeatability testing, samples of each tested organism's concentrated and diluted suspension were analyzed repeatedly and the *RSD* from 10 repetitions was less than 30% (Table 2). As *RSD*s for all remaining microorganisms were also in accordance with prescribed criteria, the repeatability of the rapid test method was confirmed.

In linearity studies, the determination coefficient was greater than 0.9 (Table 3). Furthermore, good correlations between RLU and theoretical cfu values were obtained also for all other tested microorganisms. In conclusion, the ATP-B method is linear across the range of the test for the pharmacopoeia recommended organisms.

Concerning equivalence, the data in Figure 1 show that the number of cells counted by the new method, for a range of

TABLE 1
Recovery for Concentrated and Diluted Suspensions of Test Microorganisms

Species	Dilution Factor (%)	Recovery (%) 10^8 cfu/mL	Recovery (%) 10^4 cfu/mL
Staphylococcus aureus	75	88	108
	50	90	86
	25	115	44
	10	170	40
Pseudomonas aeruginosa	75	94	88
	50	95	116
	25	111	120
	10	220	115
Escherichia coli	75	80	70
	50	90	75
	25	105	110
	10	116	111
Bacillus subtilis	75	75	78
	50	86	80
	25	99	104
	10	190	100
Aspergillus niger	75	96	100
	50	105	116
	25	126	184
	10	170	150
Candida albicans	75	90	107
	50	95	107
	25	100	115
	10	170	135
Mean (%)		115.7	102.4

TABLE 2
Repeatability for Concentrated and Diluted Suspensions of Test Microorganisms

Species	Relative Standard Deviation (%) 10^8 cfu/mL	Relative Standard Deviation (%) 10^4 cfu/mL
Staphylococcus aureus	24.6	23
Pseudomonas aeruginosa	24.9	26
Escherichia coli	15	22.3
Bacillus subtilis	19.1	26.2
Aspergillus niger	18.3	23.7
Candida albicans	20.9	29.9

TABLE 3
Individual Results Obtained by the ATP-B Method from a Serially Diluted Suspension of Test Microorganisms

Species	Theoretical Value (cfu/mL)	Average log RLU ($\pm S_{xy}$)[a]	Linear Regression
Staphylococcus aureus	10^7	6.9165 (± 0.0540)	r^2 = .993[b]
	10^6	5.9566 (± 0.0225)	S_{xy} = .188[c]
	10^5	4.9056 (± 0.0447)	
	10^3	2.9289 (± 0.0242)	
	10^2	2.4341 (± 0.1018)	
Pseudomonas aeruginosa	10^7	6.9284 (± 0.0666)	r^2 = .965
	10^6	5.4910 (± 0.1103)	S_{xy} = .439
	10^5	4.5403 (± 0.0382)	
	10^3	2.4801 (± 0.0987)	
	10^2	2.1417 (± 0.0677)	
Escherichia coli	10^7	5.6138 (± 0.0753)	r^2 = .973
	10^6	4.8748 (± 0.1104)	S_{xy} = .329
	10^5	4.0261 (± 0.0427)	
	10^3	1.9713 (± 0.0548)	
	10^2	1.6971 (± 0.0632)	
Bacillus subtilis	10^7	6.6615 (± 0.2533)	r^2 = .946
	10^6	6.2237 (± 0.2370)	S_{xy} = 0.543
	10^5	4.9231 (± 0.1718)	
	10^3	2.8354 (± 0.9646)	
	10^2	2.1140 (± 0.0837)	
Aspergillus niger	10^5	3.7364 (± 0.0977)	r^2 = .993
	10^4	3.0797 (± 0.0561)	S_{xy} = .188
	10^3	2.6532 (± 0.3859)	
	10	2.0183 (± 0.0473)	
	1	2.0513 (± 0.1275)	
Candida albicans	10^7	5.9918 (± 0.1091)	r^2 = .957
	10^6	4.8839 (± 0.2415)	S_{xy} = .353
	10^5	4.5038 (± 0.1639)	
	10^3	2.6417 (± 0.1796)	
	10^2	2.5802 (± 0.1711)	

[a]The data are expressed as a $\log_{10}$ value of the mean obtained from five independent repetitions of bioluminescent measurements.
[b]Determination coefficient.
[c]Standard deviation.

different microorganisms, was equivalent to the number of cells seen with the plate count method. These studies also indicate that, for the various organisms tested, except *A. niger*, and *C. albicans*, the system sensitivity for direct enumeration is in the region of 100 cells/mL. This is substantially more sensitive than other methods for nonfilterable products (Škof et al., 2004; Vine & Bishop, 2005). Statistical evaluation using *t*-test for paired data also confirms the preliminary results (Table 4), as we found no significant difference at the significance level α = .05. Furthermore, dual-logarithmic regression has been

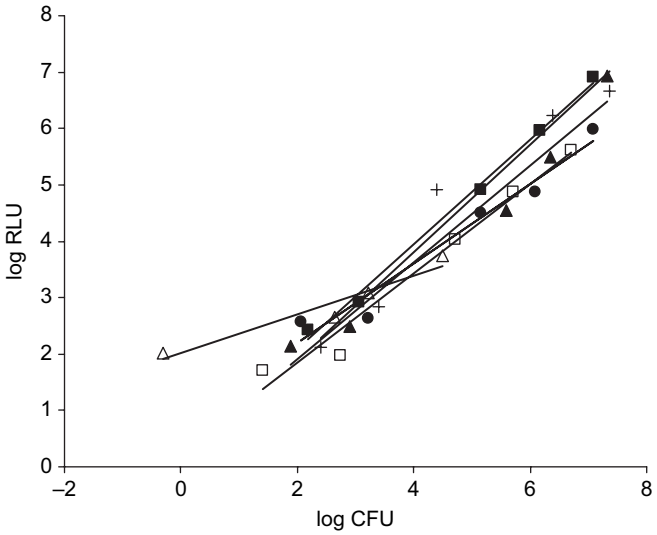

FIGURE 1. Bioluminescence measurement of ATP extracted from *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Aspergillus niger,* and *Candida albicans*. Species: (■) *S. aureus*, (▲) *P. aeruginosa*, (□) *E. coli*, (+) *B. subtilis*, (●) *C. albicans*, and (Δ) *A. niger*.

calculated, taking the log number of microorganisms obtained by the ATP-B method and the log number of microorganisms obtained by the plate count method. The results from *t*-test for slope and intercept show that the slope is statistically not different from 1 and the intercept is statistically not different from zero, and that most regression lines go through the origin of the scatter-plot system. Only in the case of *A. niger* and *C. albicans*, calculated *t*-values reach the tabulated *t*-values at the significance level $\alpha = .05$ meaning the method is less applicable to fungi, especially *A. niger*, which interferes with detection of the light emission. This is in accordance with results from previous study (Connolly et al., 1994; Venkateswaran, Hattori, La Duc, & Kern, 2003), which showed that the ATP-B method was not applicable to *Aspergillus*. In fungi, microbial ATP extraction poses additional challenges as filamentous fungi possess exceptionally robust cell wall. New ATP extraction strategies specific to fungi have been, however, elaborated (Rakotonirainy, Heraud, & Lavedrine, 2003) and extraction at 100°C with dimethyl sulfoxide (DMSO) in a Tris-acetate–ethylenediaminetetraacetic acid (EDTA) buffer proved to be the best method. It is obvious that different microorganisms act differently and demand specific approach concerning ATP-B method in such cases.

Results obtained in validation for all tested microorganisms, except for *A. niger*, where a special approach is needed, meet the validation criteria in that they give equivalent or better accuracy, repeatability, and linearity than the pharmacopoeia-recommended methods, which means a good potential for PET. The method was then applied to more complex experimental pharmaceutical formulation for further investigation.

In preservative efficacy testing, short-time analyses are not the most important criterion. For a full test according to pharmacopoeia, the total test duration is 28 days, which means that obtaining individual sample results within 1–4 h is of less importance. By contrast, the economy of the method in materials and labor costs is a prime consideration. In this aspect, rapid methods enable analysis of the number of test microorganisms in shorter time requiring less material, as well.

To prove the capability of the ATP-B method to follow the main effects of different preservative concentrations on a number of test microorganisms, we chose PBS, hydrogel and nasal spray as the model systems representing various liquid and semisolid preparations in pharmaceutical manufacture. We found as shown in Figures 2, 3, and 4, the method is capable of following different levels of test microorganisms' concentration in all media. Furthermore, the results in Figures 2 and 3 also indicate a good dose/response relationship for all tested microorganisms and all concentrations of MHB, which indicate increasing activity with increasing concentration and contact time in PBS and hydrogel. As shown it is possible with ATP-B method to follow the effect of preservative on the viability of test microorganisms in semiquantitative way as good, satisfactory, and poor. Post-hoc Scheffe test confirmed this conclusion indicating, however, that the effect is most pronounced in PBS. The reason is probably because there are less interfering substances present in this case, suggesting that it may be necessary to determine individual dose/response correlation curves for preservative systems used in different pharmaceutical formulations. It can be seen from Figure 1, as well, that the sensitivity of the method in PBS is of the order of 100 cfu/mL, which is adequate for current PET methods in which an inoculum of 10^6 cfu/mL is used for assessing a 3 log reduction.

Results in Figure 4 illustrate that the ATP-B method is applicable also for a complex semiliquid formulation, which was selected for this study because it is particularly difficult to handle. Therefore, compliance with EP requirements for topical pharmaceutical preparations was investigated and compared with that of the pour-plate method. According to the EP requirements, a topical preparation is effectively preserved if the number of bacteria recovered per mL is reduced by a factor of 10^2 within 48 h of challenge and a factor of 10^3 within 1 week of challenge, with no subsequent increase at 28 days and the number of fungi, recovered per mL is reduced by a factor of 10^2 within 2 weeks of challenge, with no subsequent increase at day 28. For each species of microorganisms, a two-way ANOVA as regards to results obtained by either method at each time interval was performed. The raw data expressed as a log number of surviving microorganisms per mL and the ANOVA results are presented in Table 5 and Figure 4. The results in Table 5 show none of the *p*-values is below the .05 level. Therefore, it can be concluded that no statistically significant difference occurs when retrocultures of a preservative test are performed either by the plate count method or by the ATP-B method. Table 5 also shows PET, performed by the plate

TABLE 4
Determination of the Number of Microorganisms in Serially Diluted Suspensions Performed by the Plate Count and the ATP-B Method

Species	Log Theoretical Value	Plate Count[a]	ATP-B[b]	Two-Way ANOVA (Significance Level $p \leq .05$)
Staphylococcus aureus	7.0	7.09342	6.91647	$p = .3274$; no significant difference
	6.0	6.17391	5.95665	
	5.0	5.14301	4.90565	
	3.0	3.06632	2.92887	
	2.0	2.17609	2.43406	
Pseudomonas aeruginosa	7.0	7.32837	6.92837	$p = .0961$; no significant difference
	6.0	6.34242	5.49101	
	5.0	5.58827	4.54027	
	3.0	2.90308	2.48006	
	2.0	1.87506	2.14169	
Escherichia coli	6.0	6.70757	5.61379	$p = .0617$; no significant difference
	5.0	5.70757	4.87477	
	4.0	4.70757	4.02609	
	2.0	2.73239	1.97129	
	1.0	1.39794	1.69710	
Bacillus subtilis	7.0	7.36642	6.66152	$p = .3340$; no significant difference
	6.0	6.38471	6.22367	
	5.0	4.38916	4.92315	
	3.0	3.39128	2.83541	
	2.0	2.40432	2.11404	
Candida albicans	7.0	7.07918	5.99179	$p = .1202$; no significant difference
	6.0	6.07918	4.88388	
	5.0	5.15986	4.50376	
	3.0	3.21748	2.64169	
	2.0	2.06069	2.58018	
Aspergillus niger	5.0	4.49415	3.73638	$p = .9298$; no significant difference
	4.0	3.20951	3.07975	
	3.0	2.64345	2.65322	
	1.0	< 1.00000	2.01826	

[a]The data are expressed as a $\log_{10}$ value of the mean obtained from growth on duplicate plates.
[b]The data are expressed as a $\log_{10}$ value of the mean obtained from five independent repetitions of bioluminescent measurements.

count method, complies with all criteria for all test microorganisms. Contrarily, ATP-B method in two cases gave results that did not comply with pharmacopoeia requirements. *P. aeruginosa* did not comply after 1 week (log reduction 2.9) and *A. niger* after 14 days (log reduction 0.9). In all other cases, the results complied with pharmacopoeial requirements. For *P. aeruginosa*, experiments showed low initial ATP levels, which may be due to incomplete ATP extraction with the reagent used. PET results for *A. niger* are in accordance with validation results, where interference of *A. niger* with detection of the light emission was shown. Results in Figures 2 and 3 and Table 5 illustrate that the ATP-B method can be used also in a complex semiliquid formulation enabling the reduction in microbiological count to be also detected in this case. At the present level of method development, the ATP-B method in most cases represents a valid alternative to plate count method for PET. However, only results obtained from pharmacopoeia preservative efficacy tests are accepted by the licensing authorities as evidence of adequate product preservation. Therefore, for final approval of the suitable preservation of the product, the plate count method should also be used.

By validation as well as by subsequent PET and equivalence testing, we have proved that ATP-B method at its current level of development has at least two major advantages, appreciated

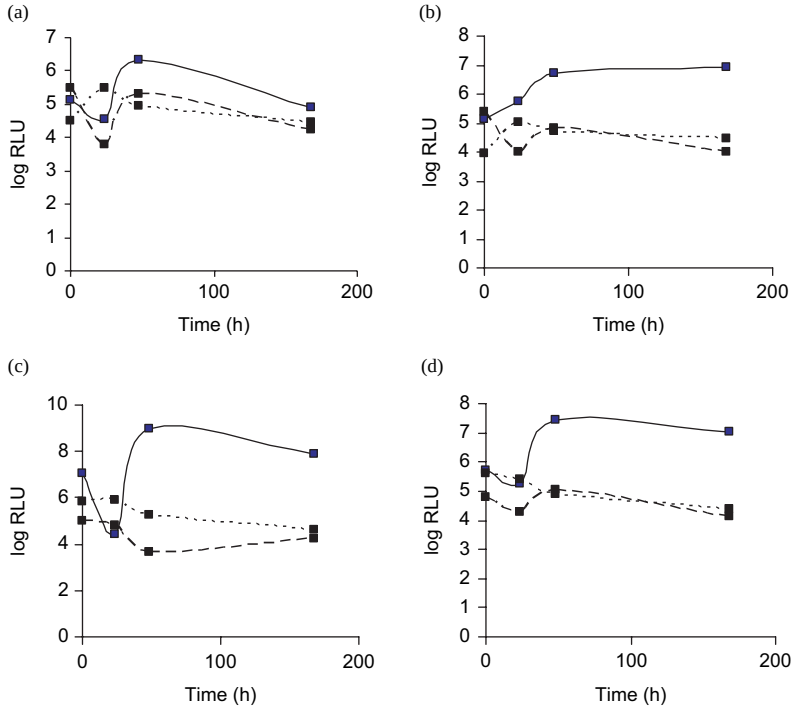

FIGURE 2. Bactericidal effect of a preservative on (A) *Staphylococcus aureus*, (B) *Pseudomonas aeruginosa*, (C) *Candida albicans*, and (D) *Aspergillus niger* in phosphate buffer solution (PBS) as determined by the ATP bioluminescence (ATP-B) method. Preservative concentrations: ——— 0% methyl parahydroxybenzoate (MHB), --------- 0.3% MHB, …… 0.6% MHB.

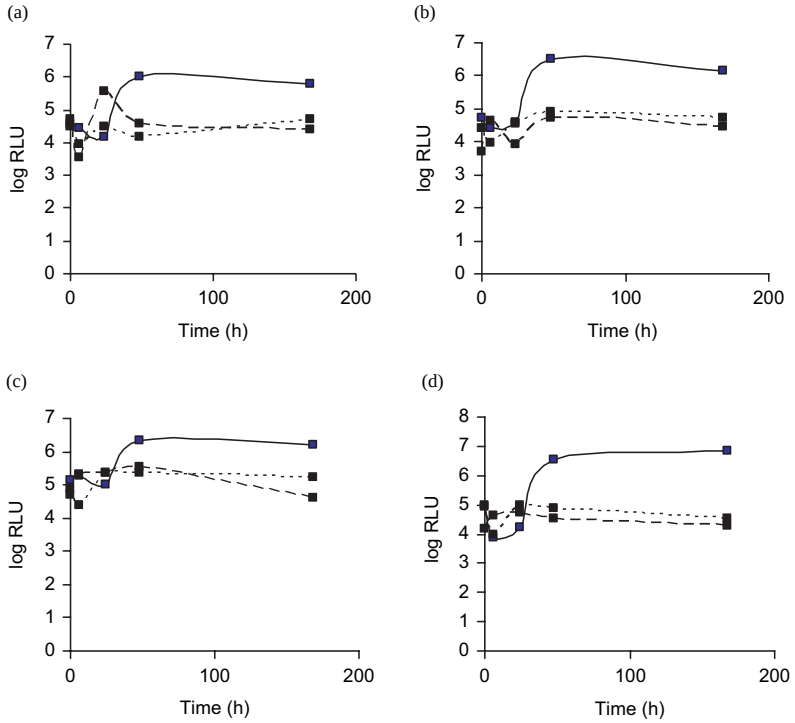

FIGURE 3. Bactericidal effect of a preservative on (A) *Staphylococcus aureus*, (B) *Pseudomonas aeruginosa*, (C) *Candida albicans*, and (D) *Aspergillus niger* in hydrogel as determined by the ATP-B method. Preservative concentrations: ——— 0% methyl parahydroxybenzoate (MHB), --------- 0.3% MHB, …… 0.6% MHB.

TABLE 5
Retroculture Results Performed by the Plate Count and the ATP-B Method from an Antimicrobial Preservative Efficacy Test with a Semiliquid Pharmaceutical Preparation

Species	Retroculture Interval	Plate Count[a]	ATP-B[b]	PhEur Compliance[c]	Two-Way ANOVA (Significance Level $p \leq .05$)
Staphylococcus aureus	0 h	7.5051	6.6532		$p = .8825$; no significant difference
	48 h	4.8692	3.3711	++	
	1 week	2.0000	3.6071	++	
	2 weeks	2.6990	3.3424		
	4 weeks	2.7404	3.2788	++	
Pseudomonas aeruginosa	0 h	6.4472	6.5357		$p = .2054$; no significant difference
	48 h	4.2076	3.6198	++	
	1 week	2.0000	4.1903	+–	
	2 weeks	1.9956	3.6435		
	4 weeks	1.3222	3.8228	+–	
Escherichia coli	0 h	5.8129	6.9208		$p = .2433$; no significant difference
	48 h	3.6472	3.3921	++	
	1 week	2.0000	3.6767	++	
	2 weeks	1.9956	3.4065		
	4 weeks	1.6628	2.9614	++	
Candida albicans	0 h	7.5798	5.3979		$p = .3213$; no significant difference
	48 h	5.1139	3.5510		
	1 week	5.3424	3.8451		
	2 weeks	4.6128	3.2430	++	
	4 weeks	0.9542	3.1461	++	
Aspergillus niger	0 h	6.2041	4.0657		$p = .0134$; significant difference
	48 h	4.9445	3.6782		
	1 week	4.6812	3.8692		
	2 weeks	4.2041	3.1903	+–	
	4 weeks	3.6021	3.0607	++	

[a]The data are expressed as a $\log_{10}$ value of the mean obtained from growth on duplicate plates.
[b]The data are expressed as a $\log_{10}$ value of the mean obtained from five independent repetitions of bioluminescent measurements.
[c]Compliance to PhEur requirements for the plate count method is represented by the first "+," whereas compliance for ATP-B method is denoted by the second "+." Noncompliance is given the symbol "–."

in pharmaceutical development. Firstly, it provides an immediate result within 1 h. The second advantage for ATP-B is its high detection sensitivity down to 100 cfu/mL for bacteria and less than 10 cfu/mL for fungi. Additionally it is also economic in labor and materials.

As stated before, a number of considerations must be taken into account when developing the ATP-B method for evaluation of pharmaceutical formulations. According to Connolly and colleagues (1993) and Dowhanick and Sobczak (1994), one of the major problems with pharmaceutical formulation is possible interference of formulation constituents with detection of the light emission. Formulation constituents can absorb or disperse emitted light, which severely limits the detection sensitivity and consequently leads to the occurence of false-positive results. Moreover, bioluminescence reagent supression and inhibition of enzymatic reaction with formulation constituents are possible. In these cases, an experimental design to evaluate product interferences with detection of the light emission should be applied.

In our study, improved confidence in the bioluminescence assay itself was achieved by standardization procedure using the addition of ATP standard solution. The ATP standard solution was diluted with demineralized water and light intensity measured. Additionally, a preserved sample was inoculated with ATP standard solution and diluted to approximately 7×10^2-fold with demineralized water. Light intensity measurements were performed for each dilution and compared with results of light intensity measurements of ATP standard solution in demineralized water. No statistically significant difference in instrument response occurs when diluting ATP standard in demineralized water or preserved sample as shown by a two-way ANOVA

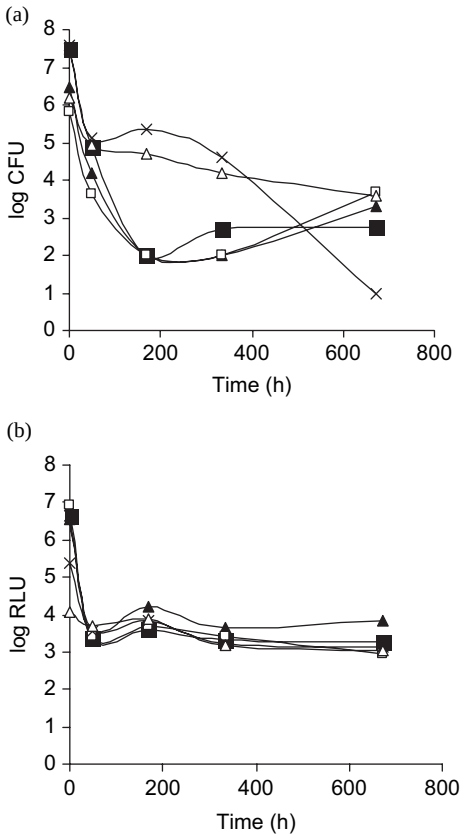

FIGURE 4. Preservative efficacy test (PET) with semiliquid formulations performed by (A) the plate count method and (B) the ATP-B method. Species: (■) *Staphylococcus aureus*, (▲) *Pseudomonas aeruginosa*, (□) *Escherichia coli*, (×) *Candida albicans*, and (Δ) *Aspergillus niger*.

TABLE 6
Comparison of ATP-B Measurements When Diluting ATP Standard in Demineralized Water and Preserved Hydrogel

Log ATP	Demineralized Water (log RLU)[a]	Preserved Hydrogel (log RLU)[a]	Two-Way ANOVA (Significance Level $p \leq .05$)
6.0	6.3222	6.1461	$p = .3658$; no
5.0	5.4914	5.1139	significant
4.0	4.4150	4.2041	difference
3.0	3.4150	3.2304	
2.0	2.4771	2.8129	

[a]The data are expressed as the mean obtained from at three independent repetitions of measurements.

at significance level $\alpha = .05$ (Table 6). *t*-Statistics for slopes at $\alpha = .05$ proved that the slopes of the regression lines were statistically not different from 1 indicating the compatibility of the enzyme system with experimental formulations. In conclusion, all experimental media were chemicaly and physicaly compatible with the enzyme system resulting in efficient sensitivity of the ATP-B method for detecting microorganisms.

To check for the occurence of false positives, we analyzed a 10% product suspensions were analyzed to determine whether they contained nonmicrobial ATP. According to producer instructions, we run two samples in parallel. One sample was tested without prior extraction, which limits the enzymatic reaction to only free ATP. The second sample was tested after extraction, which enables the detection of the total ATP. These results were supported with the determination of the background counts by measuring RLU of diluent solution and experimental formulations, as well. The discrimination value between the microorganism signal and the background of the tested media was in accordance with the calculated value, that is approximately 100 RLU, indicating no false positives can be expected.

From the results and experience obtained from our study, it can be concluded that ATP-B method at their present level of development represents a valid alternative method for PET in pharmaceutical development. Results with ATP-B method indicate that the method is applicable to a range of bacterial and yeast test strains with a detection sensitivity equivalent to the plate count method. The method is less applicable to filamentous fungi, which needed a special care. With ATP-B method, we can follow and compare the effect of preservative, as well, as its concentrations on the viability of test microorganisms. With ATP standardization procedure and enzyme compatibility testing, improved confidence in the bioluminescence results was achieved.

CONCLUSIONS

Based on the results of our study, we can conclude that the ATP-B method is of great value in screening pharmaceutical formulations. The results obtained are supported with the validation of the ATP-B method following guidelines PDA Technical Report No. 33 “Evaluation, Validation, and Implementation of New Microbiological Testing Methods.” At the present level of method development, ATP-B was found to be sensitive enough for PET, as the lower limit of detection is of the order of 10^2 cfu/mL. By this approach, therefore, a greater range of preservative combinations and concentrations could be evaluated and the most effective selected. On the basis of preservative concentrations, types, and combinations, a prediction of preservative efficacy in a new formulation could be made. Thus, such predictions must be validated on a formula-by-formula basis because of possible inherent preservative actions and synergistic-antagonistic activities of pharmaceutical components. We can conclude that this method offers another very useful application of ATP bioluminescence for the pharmaceutical industry, as the method turned out to be as best in screening pharmaceutical formulations. Further investigations are being carried out to evaluate

the use of ATP-B method for preservative efficacy screening testing with a range of preservative agents in aqueous and cream formulations. The main advantage of this rapid method is the reduction of operating time and costs. Consequently, the important reduction of working hours yielded by the use of an automated system would allow thus to perform the challenge test with additional organisms, including in-house isolates and objectionable microorganisms, which are an emerging problem in oral pharmaceuticals due to the increasing number of immuno-compromised patients.

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