
Detection and Comparison of Low Level Microorganism Contamination in Pharmaceutical Products Utilizing ATP Bioluminescence Methodology

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Abstract

As health care companies strive to increase testing efficiencies while sustaining a high level of product quality, new analytical technologies offering greater sensitivity, specificity and speed are becoming essential tools in the evaluation of materials at all stages of pharmaceutical manufacturing processes. Adenosine triphosphate (ATP) bioluminescence methodology can reliably detect microbial contamination in pharmaceutical samples within 48 hours, reducing analysis time significantly. This study evaluated a rapid ATP bioluminescence method for pharmaceutical product analysis. The research comprised the following phases: (1) assessment of indigenous ATP to determine baseline relative light unit levels; (2) detection and comparison of broad spectrum, low level microbial contamination; and (3) statistical analysis of data to determine if genera specific patterns exist. Products tested with the bioluminescence assay were prepared utilizing the following steps: (a) initial product dilution using TAT broth; (b) inoculation of product suspensions with ATCC microorganisms and environmental flora; and (c) 48 hour incubation of suspensions at 30-35° C and 20-25° C. Following incubation of controls and inoculated samples, the bioluminescence assay was initiated by aseptically transferring 50 uL aliquots (in duplicate) to the bioluminescence chamber. Automated assay control resulted in the injection of ATP releasing agent (Celsis LuminEX™) and luciferin-luciferase. Indigenous ATP levels for pharmaceutical samples resulted in a 1500 to 4000 relative light unit range. All USP Microbial Limits Test indicator organisms were detected in product suspension using the bioluminescence system within 48 hours. Statistical analysis indicated that a genera specific relationship exists between initial contamination levels and luminometer measured microorganism ATP. Bioluminescence provides a potential rapid method to consistently assure a level of product quality by qualitatively screening for low level viable contaminants and therefore has potential as a useful tool in microbiological testing of materials in the health care industry.

Introduction

Although there have been significant steps to evaluate and implement rapid microbiology methodologies in the pharmaceutical industry, traditional plate count methods are still the mainstay for enumeration of microorganisms in many Quality Control laboratories. An alternative option to the traditional USP Microbial Limits Test methodology is the measurement of microbial viability using cellular ATP. "Bioluminescence" provides microbiologists with a rapid screening tool for the detection of potential product contaminants. Bioluminescence is considered a rapid microbiology technology compared to traditional methods of culturing organisms from product.

Traditional methodologies utilized in Quality Control microbiology laboratories are often time consuming and costly. Rapid technologies, such as ATP-Bioluminescence, are gaining more acceptance in the health care industry, facilitating a significant reduction in sample through-put time while sustaining or elevating the essential level of quality. Pharmaceutical companies have been slow to implement rapid microbiology methodologies due to uncertainty of regulatory expectations. Extensive work performed at Hoffmann La Roche (QM Microbiology; Nutley, New Jersey) offers support for implementation of rapid ATP-Bioluminescence as a routine qualitative screening tool for determining the microbiological quality of raw materials, active pharmaceutical ingredients (API's) and final dosage forms.

Automated systems can offer a high level of sensitivity and reliability, which meet the challenges of increased Pharmaceutical industry demand (efficient and effective testing). The purpose of this developmental research was to evaluate and optimize rapid detection of low-level microorganism contamination in Pharmaceutical samples (final dosage forms, active pharmaceutical ingredients and raw materials) utilizing adenosine triphosphate (ATP) bioluminescence methodology.

ATP is the energy source of all viable organisms and is essential to cellular metabolic pathways. Many luminescent organisms, such as the firefly, contain a species-specific thiol containing the oxido-reductase enzyme luciferase. This enzyme catalyses the oxidation of a light emitting substance, luciferin, in the presence of ATP and other factors. The light generated with the bioluminescence assay indicates the presence of active ATP and hence viable microorganisms. The relative light unit measurement is proportional to the amount of ATP present. Pharmaceutical samples can be effectively and efficiently evaluated for the presence of viable microorganisms with the measurement of ATP using light produced from the luciferin-luciferase reaction.

To isolate organism ATP, a chemical releasing agent (Celsis LuminEX™) is used to increase the permeability of the cell membrane, allowing the release of ATP into the extracellular environment. The injection of purified luciferin-luciferase (Celsis LuminATE™), reacting with the ATP to produce light, indicates the presence of viable microorganisms in pharmaceutical material. When a microbial cell dies, ATP production ceases and the pre-existing ATP is destroyed rapidly by cellular ATPase and various phosphatases. Therefore, only viable organisms present in pharmaceutical products will produce ATP that can be detected using the bioluminescence assay.

Materials and Method

Instrument and reagents

The rapid bioluminescence assay was performed using the Celsis Advance™ Luminometer and a Personal Computer. RapiScreen reagent kits (600 assays) consisting of Celsis LuminATE™ (HS) and Celsis LuminEX™; wash and rinse kits and ATP positive control kits were utilized in this study.

Sample preparation and test conditions

All pharmaceutical test dilutions were prepared using TAT broth and maintained in incubators adjustable to 30-35° C and 20-25° C. A freezer capable of maintaining -18° C or below was utilized for the ATP standard. A 10 uL aliquot of ATP standard was used for daily Quality Control of the bioluminescence assay.

The following stock cultures were used for inoculation of test samples

Staphylococcus aureus ATCC 6538; *Pseudomonas aeruginosa* ATCC 9027; *Escherichia coli* ATCC 8739; *Salmonella typhimurium* ATCC 14028; *Bacillus subtilis* ATCC 6633; *Candida albicans* ATCC 10231; *Aspergillus niger* ATCC 16404; *Enterobacter cloacae* ATCC 13047; and environmental isolates.

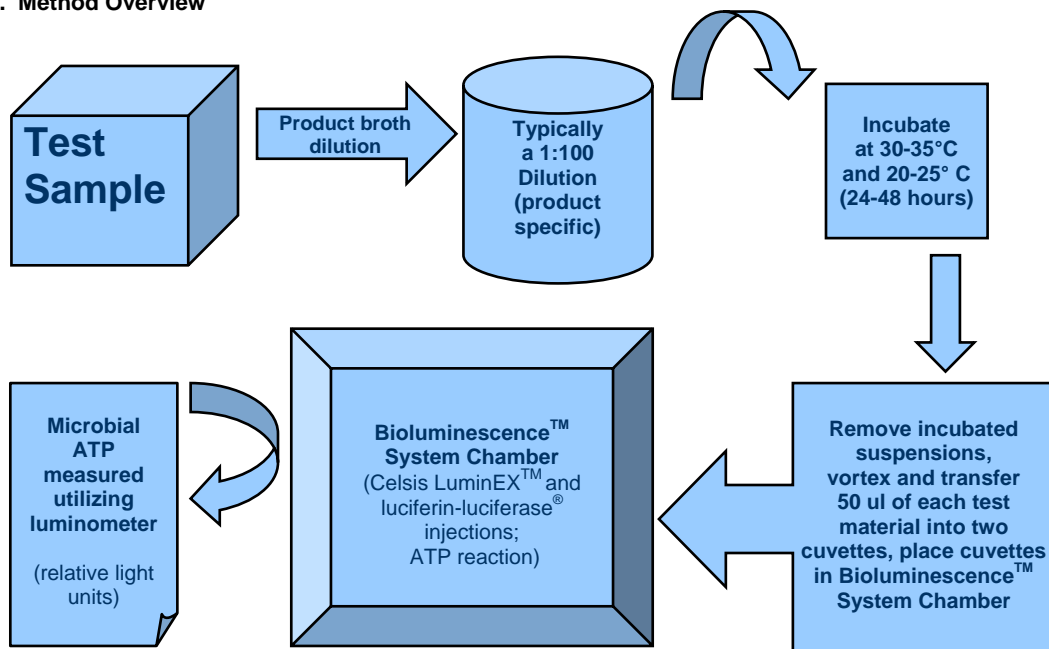
Bioluminescence assay

24-48 hour incubated test materials were transferred to cuvettes (dimension 55mm x 12mm) in duplicate. 50 uL aliquots were analyzed by the luminometer using Celsis LuminEX™ (releasing agent) and Celsis LuminATE™ (Luciferin-Luciferase). A negative TAT broth control was measured to determine a positive cutoff value for test material assayed. Test material measured at two times (or greater) the negative broth control were considered positive for the presence of viable microbial ATP.

Samples measuring less than two times the negative control (as determined by the luminometer in relative light units) were considered negative for viable microbial ATP and therefore recorded as no contamination present in test samples.

Test samples exhibiting positive luminometer signals are evaluated by traditional test methodologies (example Microbial Limits Test).

Figure 1. Method Overview



Results

Figure 2. Phase 1 (Sample Effects): measurement of indigenous ATP levels in pharmaceutical samples using a bioluminescence luminometer; 6 representative samples were selected for the assay and a total of 18 lots were screened.

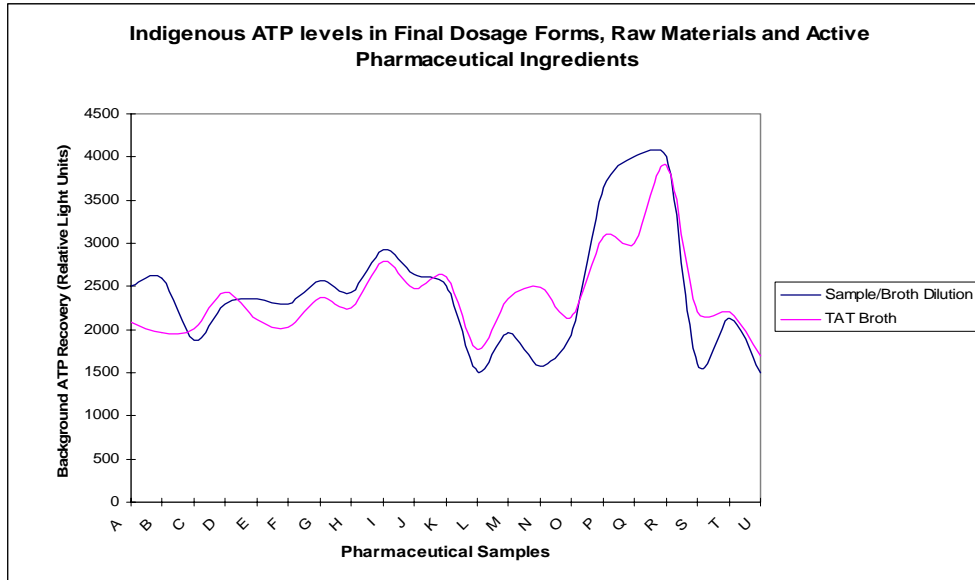


Table 1. Phase II (Inoculation Studies): Detection of Low Level Contamination in Pharmaceutical Samples Using ATP Bioluminescence

| Organism | CFU/mL | Sample A | Sample B | Sample C |
|--|--------|-------------------------|-------------------------|-------------------------|
| | | Avg. ATP (Log of RLU's) | Avg. ATP (Log of RLU's) | Avg. ATP (Log of RLU's) |
| <i>S. aureus</i> (ATCC 6538) | 3 | 6.5 | 6.7 | 7.2 |
| <i>P. aeruginosa</i> (ATCC 9027) | 2 | 6.7 | 5.8 | 7.3 |
| <i>E. coli</i> (8739) | 2 | 6.6 | 6.5 | 6.5 |
| <i>S. typhimurium</i> (14028) | 2 | 6.4 | 6.1 | 6.6 |
| <i>B. subtilis</i> (ATCC 6633) | 3 | 6.8 | 7.0 | 7.3 |
| <i>C. albicans</i> (ATCC 10231) | 3 | 5.5 | 7.0 | 7.2 |
| <i>A. niger</i> (ATCC 16404) | 6 | 7.0 | 4.1 | 6.9 |
| <i>Micrococcus species</i> (environmental isolate) | 51 | 6.7 | 6.9 | 7.0 |
| <i>E. cloacae</i> (ATCC 13047) | 14 | 5.7 | 6.6 | 5.4 |

Pharmaceutical samples A, B and C were selected for the ATP Bioluminescence assay. All data are in log form and resulted from Bioluminescence measurements except initial colony forming units. **CFU:** initial (colony forming units) inoculum used to spike samples. **AVG ATP:** the average microbial ATP measured with a luminometer, in relative light units (RLU's), from pharmaceutical samples A, B and C (run in duplicate). In addition to the data in Table 1, a negative control (product + broth dilution) was used in the bioluminescence assay and measured between 1500 to 4000 relative light units. Samples are considered positive when the luminometer measurement is equal to or greater than two times the negative bioluminescence signal.

Figure 3. Phase II (Inoculation Studies): Pharmaceutical samples A, B and C spiked with low level microorganism inoculums, incubated and assayed following the ATP bioluminescence protocol.

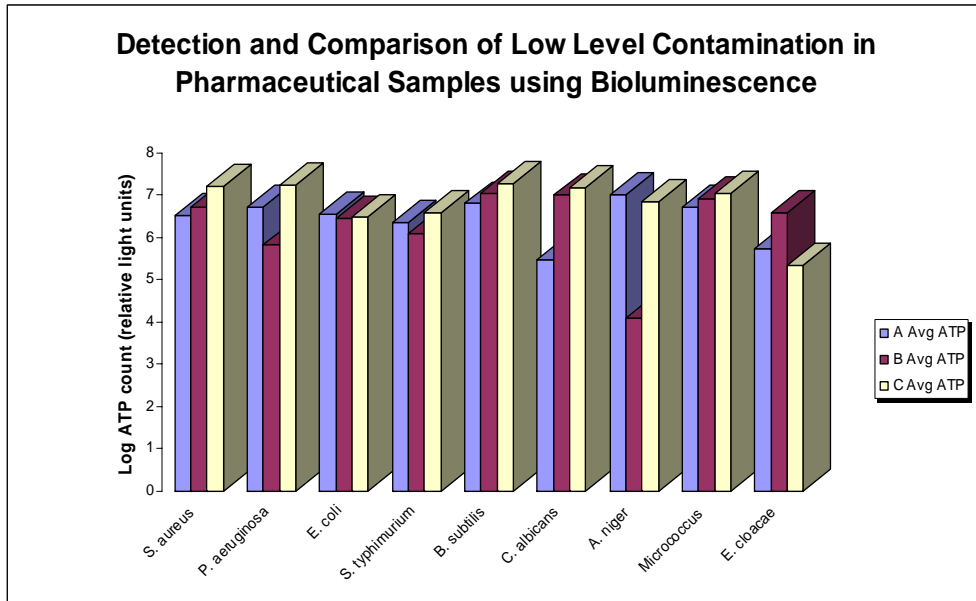
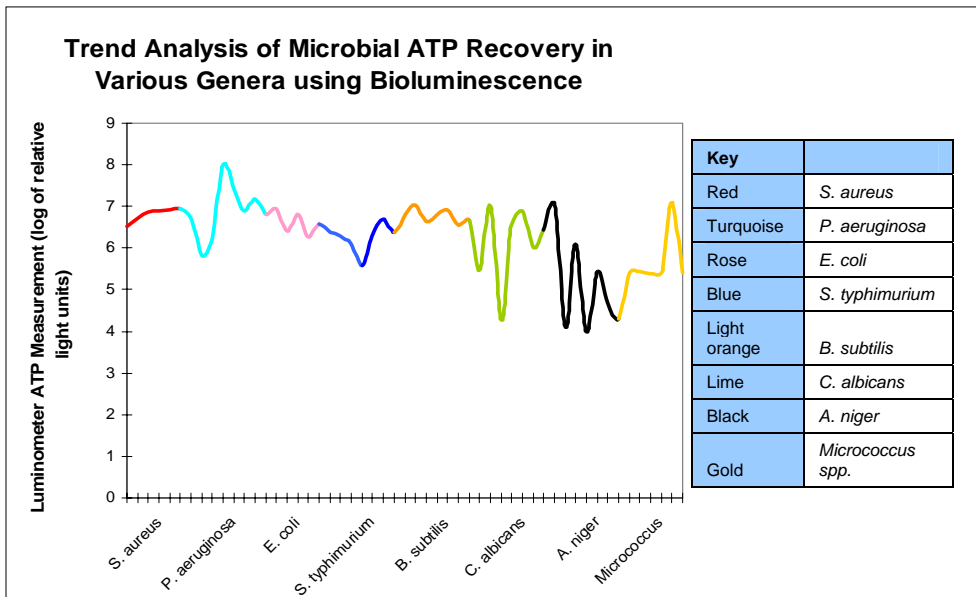


Figure 4. Phase III (Genera Specific Patterns): ATP recovery of low level microbial contamination in 15 representative Pharmaceutical samples.



ATP Bioluminescence Technology



Discussion

In Phase I of this study, indigenous ATP levels for final dosage forms, active pharmaceutical ingredients (API's) and raw materials were determined using a Celsis Advance luminometer. No significant inhibition or enhancement of the luciferin-luciferase reaction was observed for TAT broth or material test dilutions. Typically, pharmaceutical test dilutions exhibit background relative light unit levels (RLU's) between 1500 and 4000 as measured with the luminometer. Two times the Luminometer measurement of non-microbial ATP in TAT broth was considered a positive signal for all test samples assayed in Phase II.

In Phase II of this study, a broad spectrum of microorganisms (ATCC and environmental flora) were detected in pharmaceutical materials within 48 hours. Low level inoculums were utilized ranging from 2 colony forming units to 51 colony forming units. After 48 hours incubation at 30-35° C, a positive signal for bacterial isolates recovered in pharmaceutical material exhibited bioluminescence values of 3.3×10^6 to 6.4×10^6 RLU's. Positive signals for fungal isolates recovered in pharmaceutical material exhibited bioluminescence values of 3.0×10^5 to 1.1×10^7 RLU's. *Micrococcus species*, selected as a representative environmental isolate, exhibited an average bioluminescence value of 2.5×10^5 RLU's. It should be noted that although *A. niger* ATP recovery exhibited the greatest variability in pharmaceutical materials, mold was reliably detected using the luminometer.

Phase III of this study assessed a trending pattern exhibited by a broad spectrum of microorganisms. Microbial ATP recovery in pharmaceutical material, measured with the luminometer, displayed genera specific patterns. The least variability was observed for *Staphylococcus* ATP. The greatest variability of ATP recovery was exhibited by *Aspergillus*. *Pseudomonas* has the potential to exhibit high ATP levels, reaching 9.9×10^7 RLU's, while *Aspergillus* ATP recovery exhibits consistent detection within 48 hours at an incubation temperature of 30-35° C.

Conclusion

This Bioluminescence study offers support for implementation of an innovative technology in the analysis and control of materials used and produced by a pharmaceutical manufacturing operation. The methodology can be utilized to rapidly and reliably detect low level viable microbial isolates in pharmaceutical materials (Final Dosage Forms, Active Ingredients and Raw Materials) within 48 hours, thus reducing traditional analysis time significantly.

Rapid qualitative screening of numerous materials along the complex manufacturing process enables strategic decision making and assures a consistent level of product quality.

ATP-Bioluminescence is recommended as a sensitive screening tool for quality control laboratories. The future for a practical application of this technology in the pharmaceutical industry is promising. This rapid method can potentially be utilized as a screening method alternative to the traditional Microbial Limits Test.