General Internal Medicine and Clinical Innovations

oat open access text

Research Article

The harmonized microbial limits test

Clyde Schultz[1,2*]

[1]Department of Biology, University of Calgary, Calgary, Alberta, Canada

[2]Biogram Inc., Ponte Vedra Beach, FL, USA

Abstract

The Harmonized Microbial Limits Test is a compendia method used by commercial and clinical/developmental laboratories to evaluate the bioburden of solid oral dosage formulations such as pills and tablets. These products are manufactured and sold worldwide as non-sterile formulations. This test has its origins in the middle part of the 20th century when final product coatings and manufacturing facility controls were being recognized as important in the pharmaceutical industry, but were in their infancy in terms of sophistication. The test was "harmonized" in 2009 and as such certain aspects of the procedure were changed to accommodate a global testing requirement. Prior to 2009, there were variations of the test in different countries which required re-testing when a given product was sold in a country different from the country that the product was originally tested in. This standardization has largely improved the work flow of products in a global market, and made it easier for laboratory specialists in different countries to resolve compliance issues when they arise.

Introduction

The Harmonized Microbial Limits Test (MLT) is used in The United States, Europe and Japan as the primary biological release test for non-sterile solid oral products intended for human use [1-4]. It is a compendia test meaning that it is published and meant to be used "as is" with little or no variation from the method. The purpose of the harmonization of the test was to avoid unnecessary testing duplication which occurred prior to the international harmonization effort in 2009 [1,2]. The data obtained from the test itself is meant to provide a level of control, and thus comfort that a minimum standard has been met relative to product bioburden and thus an assurance of a level of manufacturing control has been maintained over the production of tablets and pills intended for sale. The test has not changed fundamentally in terms of execution since its inception. In one sense it can be argued that technology from the last century is being used to release product made in the 21st century, and thus serve as a microbiological control for release of product for human use. The MLT is designed to provide quantitative information in terms of numbers and qualitative information of viable organisms present in samples. Depending on the media used total aerobic microbial counts, total yeast and mold counts and a qualitative analysis for *E. coli* are performed at minimum. There are procedures for qualitative determination of other organisms as well. But they are seldom used unless requested, and these are country specific.

Industry uses this test because it is simple and inexpensive to perform. It is also recognized globally, and the data obtained are accepted by most countries in the world. It is also subject to laboratory error if not performed by competent technical staff. Tablets and pills are manufactured in the absence of water. Since a minimum of water is necessary for survival of vegetative bacterial cells, any delay in testing results in negative test results, even if there was contamination initially [5]. Practically, most bioburden dies off during actual manufacture of the tablet. This is because of the massive dust generated during manufacture (operators wear personal protective equipment, including respirators), temperature in the manufacturing areas and as mentioned, lack of water necessary for growth and the toxic composition of many of the excipients and active pharmaceutical agents used to produce solid oral dosage formulations.

The harmonized microbial limits test

There are four parts to the MLT. These will be discussed in turn.

System suitability: The system suitability part of the test is performed initially to determine if the product being evaluated will enhance or inhibit bacterial or fungal growth. This is the "control" aspect of the test and once successfully conducted does not have to be performed again in the same laboratory, as long as there are no formulation changes with the product. The concept is to place a given number of organisms (<100 colony forming units (cfu)) in a confined space with diluted product. The inoculum volume should not increase the overall sample volume by more than 1%. There are five microbes that are used in this phase of the test. The organisms that are evaluated are *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Bacillus subtilis (6633), Candida albicans* (ATCC 10231) and *Aspergillus brasillensis* (ATCC 16404). All five organisms are evaluated on Soybean Casein Digest Agar. *Candida* and *Aspergillus* are also evaluated on Saboraud Dextrose Agar. The strain numbers are important to provide standardized isolate identification relative to the test. The product is put into solution or suspension beginning at a 1/10 dilution of product (10grams) to diluent. The diluent is most often a phosphate buffered saline (pH 7.2). Should a product exhibit inhibition or enhancement properties, serial dilutions of the product are made to decrease the inhibitory/enhancement effect. Other ways of reducing enhancement/inhibition include the use of neutralizers, including lecithin. The results obtained following incubation periods

Correspondence to: Dr. C. Schultz, Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4 Canada, Tel: 403-220-5278; E-mail: cls_5us@yahoo.com

Key words: *bioburden, bacteria, fungi, compendia, non-sterile*

Received: August 01, 2016; **Accepted:** August 06, 2016; **Published:** August 10, 2016

are compared to PBS controls, which have no product exposure but are conducted in parallel with the product test. The acceptance criteria are 50 to 200% of the controls from both system suitability and product testing (below). That is both product test and control test must be within that range relative to each other to be considered passing. In that sense a product cannot "fail" the system suitability aspect of the test. The purpose is to "range" in the product starting at the 1/10 dilution. Most products range in suitability at this dilution. However, it is possible to achieve results at a higher dilution. Should a product be at an extreme dilution range (1/500 or greater), it is assumed that the product has inherent anti-microbial properties and would have no, or a reduced bioburden load upon ingestion. Whatever dilution is used to achieve within specification results, this carries forward to the actual product testing described below.

Total Viable Aerobic Count (TAMC): Once system suitability is determined, the product bioburden can be evaluated, that is to determine the amount of bacterial contamination that is in a given amount of sample. The data that are obtained are used by manufacturers to release products to the market for sale and use. The product is diluted in buffer to the concentration shown in the system suitability part of the test, and then usually added (in 1 mL amounts) to a petri plate. There are other filtration or spread plate methods used but these are less frequent than direct plating of sample. This plate is then filled with soybean casein digest medium (TSA). Once the media are solidified they are placed (inverted) in a 35°C incubator for 3-5 days to allow for bacterial growth. TSA is a general purpose medium for supporting the growth of most bacterial species. The growth limit is $\leq 10^3$cfu/gm, or 2,000cfu/gm [6]. Industry will often use ≤1000cfu/gm as the limit.

Total Viable Yeast and Mold Count (TYMC): This test is conducted in the same fashion as the total aerobic count. There are however a few differences. The first is the medium used. Saboraud Dextrose Agar (SDA) is used as the growth medium. Second the incubation temperature is set at 25°C to better promote fungal and mold growth. Third the incubation time is increased to 5-7 days to provide for a longer growth cycle which mold and fungi require. The limit is $\leq 10^2$ cfu/gm [6]. Industry will often use ≤100 cfu/gm as the release criteria.

The above tests are considered to be quantitative in nature. Should plate counts be outside the specified limits, the product will fail the test, unless a manufacturing or laboratory investigation is conducted showing that an error was made in the testing protocol.

Test for specified organisms (USP <62>) is a qualitative test. This aspect tests for what are referred to as specified organisms. Prior to 2009, there were four different organisms that required assay. After harmonization, only the examination of solid oral products for *E. coli* was required. There are some countries such as Australia that require the test for Salmonella, unless the sponsor can show that all the excipients that make up the product formulation are of a non-natural origin. But this is not the routine for most countries. System suitability is also performed on this aspect of the test. *E. coli* and other bile tolerant species are evaluated on MacConkey Agar. *E. coli* growth on MacConkey agar is unique and is characterized by a deep red, brick type appearance. If other types of colonies are observed on MacConkey agar, they are not evaluated and the product passes. There is no routine identification of organisms or further evaluation done. However, if *E. coli* is observed on the plates and then confirmed by some identification technique, then the product fails the test. The MLT cannot be repeated unless a compliance investigation is conducted and there is evidence of a laboratory error.

Interpreting results

As mentioned above, once system suitability for a given product has been established this "control" does not have to be repeated at the same testing center as long as the product formulation does not change. If the product fails the test, it cannot be repeated. The product lot must be discarded, unless an investigation is conducted and there is demonstrable evidence that there was an actual testing error. Should this scenario occur, the product may be re-tested and the initial results discarded.

Issues with the MLT test

The microbial limits test has several issues associated with it. It is an old test that has been used for decades to evaluate product, with little substantial positive procedural change.

One major issue is that of sample size. All compendia require for evaluation is a single 10 gram sample, regardless of the amount of bulk or final product available. So a 10gram sample could come from a 50kg drum. This is not statistically significant, by any except the most optimistic measure. While the media and incubation temperatures used are considered acceptable by most people skilled in the art, the incubation times are shorter than would be expected by that same group. Most people skilled in the art would favor incubation times of several weeks for mold or fungal organisms to grow due to the slower metabolic rate than fast growing bacteria.

The test does not assay for anaerobic organisms (or virus). There is no favorable media used and no anaerobic incubation condition met.

The strains used to evaluate for system suitability are laboratory strains, not wild-type strains. While standardization is essential to allow for testing consistency over sites and products, some reasonable wild type strain evaluation would enhance patient safety, which is the stated goal of all compendia tests. This is especially true in emerging nations.

System suitability results and thus potential product results may vary between laboratories. This is in many respects the biggest issue with the test. A given laboratory may obtain a system suitability result at one dilution, but another laboratory may obtain a different result. Since these control results are allowed to vary between laboratories a given product may be ultimately tested at different dilutions. The ultimate reasons may include slight variations in technique among operators who perform the test, media sources and water sources. All of this is acceptable to regulatory agencies around the world.

Compendia tests are not validated in the classic sense, they are not usually conducted in triplicate. There is only verification required, which is defined for the MLT as one successful system suitability and associated product evaluation. That is if all aspects of the test are successful commercial product lots can be evaluated and released for sale based essentially on the results of a single replicate.

Conclusion

The Harmonized Microbial Limits test is the globally standardized method for determining the bioburden of a given raw material or final product formulation of oral solid non-sterile product. It will continue to be used in industry as defined by compendia to release product for human use. It is simple and in-expensive. Part of the test is quantitative and part is designed to identify if a specific organism such as *E. coli* is present. *E. coli* is the most common organism of the human digestive tract. So to specifically look for that organism is logical on some level.

However, it is assayed for in water supplies worldwide and especially in the developed world. The presence of *E. coli* in a sample could signal either a human or facility water issue, and thus is serious. Prior to 2009, other organisms were evaluated as well. In an effort to reduce the burden on industry, these organisms were dropped, and not required further.

References

1. Food and Drug Administration, HHS (2009) International Conference on Harmonisation; guidance on Q4B Evaluation and Recommendation of Pharmacopoeial Texts for Use in the International Conference on Harmonisation Regions; Annex 4A on Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests General Chapter; availability. Notice. *Fed Regist* 74: 15991-15992. [Crossref]

2. Food and Drug Administration, HHS (2009) International Conference on Harmonisation; guidance on Q4B Evaluation and Recommendation of Pharmacopoeial Texts for Use in the International Conference on Harmonisation Regions; Annex 4B on Microbiological Examination of nonsterile products: Tests for Specified Microorganisms General Chapter; availability. Notice. *Fed Regist* 74: 15989-15990. [Crossref]

3. EP 7, Chapter 2.6.12, Microbial examination of non-sterile products (Total Viable aerobic count).

4. EP 7, Chapter 2.6.13, Microbial examination of non-sterile products (Test for specified microorganisms).

5. Mossel D, Corry J, Struik C, Baird R (1995) Essentials of the microbiology of foods: a textbook for advanced studies. Chichester (England): John Wiley and Sons. P 699.

6. Food and Drug Administration, HHS (2009) International Conference on Harmonisation; guidance on Q4B Evaluation and Recommendation of Pharmacopoeial Texts for use in the International Conference on Harmonisation Regions; Annex 4C on Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use General Chapter; availability. Notice. *Fed Regist* 74: 15992-15993. [Crossref]