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Uncertainty of quantitative microbiological methods of pharmaceutical analysis

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ABSTRACT

The total uncertainty of quantitative microbiological methods, used in pharmaceutical analysis, consists of several components. The analysis of the most important sources of the quantitative microbiological methods variability demonstrated no effect of culture media and plate-count techniques in the estimation of microbial count while the highly significant effect of other factors (type of microorganism, pharmaceutical product and individual reading and interpreting errors) was established. The most appropriate method of statistical analysis of such data was ANOVA which enabled not only the effect of individual factors to be estimated but also their interactions. Considering all the elements of uncertainty and combining them mathematically the combined relative uncertainty of the test results was estimated both for method of quantitative examination of non-sterile pharmaceuticals and microbial count technique without any product. These data did not exceed 35%, appropriated for a traditional plate count methods.

1. Introduction

When reporting the result of a measurement, it is obligatory that some quantitative indication of the quality of the result be given so that those who use it can assess its reliability. The concept of "uncertainty" as a quantifiable attribute is relatively new in the history of measurement, although "error analysis" have long been a part of the practice of metrology (ISO/IEC, 2008). The formal definition of the term "uncertainty of measurement" is as follows: parameter, associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand (International Organization for Standardization, 1993). This parameter is usually expressed as the standard deviation or the relative standard deviation, which is also referred to as the coefficient of variation.

With some chemical methods it is possible to assume the general validity of method-specific repeatability and reproducibility parameters determined in collaborative method performance studies. There are reasons why this approach is likely to be less successful in microbiology. One is the unpredictable colony number that varies from case to case and is usually the main cause of uncertainty. The other is the nature of samples. Uncertainty of a test result depends too much on the conditions under which a test is made. The best approach in microbiology seems to be to compose an uncertainty estimate from the separate uncertainties of the unit operations of the procedure (Seppo Niemelä, 2002).

The methodology of microbiological examination of non-sterile pharmaceutical products is designed primarily to determine whether a substance or preparation complies with an established specification. The tests described in pharmacopoeia allow quantitative enumeration of mesophilic bacteria and fungi and determination of the absence or limited occurrence of specified microorganisms that may be detected under aerobic conditions (European pharmacopoeia, 2014). The routine analysis procedure consists of several main stages: careful mixing or homogenization of the sample, suspending a measured portion of it in an aqueous solution, transferring the aliquots to proper solid and liquid media for quantitative and qualitative determination of contaminants, incubation in aerobic mesophilic conditions; interpretation of the results.

The total uncertainty of quantitative microbiological test result consists of several components. It is possible to identify some causes of variability, for instance:

- nature of sample (homogeneity, which influence the spatial distribution of the microorganisms; antimicrobial activity of medicine etc.);
- method of analysis (pour-plate, surface-spread method);
- growth promotion properties of culture media;

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- reading, interpreting of the result (individual features of experts).

Inoculum standardization techniques provide a quantity of microorganism for routine use in microbiology to demonstrate the efficacy of testing methods and culture media. It is normally prepared by diluting a culture of test strains to obtain a suspension that contains an estimated number of colony-forming units per milliliter. The microbiology industry requires a precise reference material for microorganisms to provide a consistent number of cells, with a reproducible amount of variation (2 standard deviations from the mean). This standard needs to be in a format that is easy to use and does not require specialized storage conditions. Existing commercially available reference materials for the microbiology industry cannot deliver this level of precision and the variability. The inaccuracy of these standards increases the potential for false results (Morgan et al., 2004).

The objective of the work was the analysis of the most important sources of the quantitative microbiological methods variability and the estimation of combined uncertainty of test result.

2. Materials and methods

The study was based on the experimental data obtained from one laboratory. Since there were several factors to be considered, data sets were investigated separately. There were analyzed some kinds of microorganisms, media, methods, formulations, inoculums standardization techniques. The reading errors and dilution factor were also estimated.

2.1. Microorganisms

Bacillus cereus,*Bacillus subtilis*, *Penicillium verrucosum*, *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus brasiliensis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*.

2.2. Media and growth conditions

trypcase soy agar and Sabouraud dextrose chloramphenicol agar (Biomerieux, France) and nutrient media for bacteria and fungi cultivation based on pancreatic fish protein hydrolysate, the formulas of which are detailed in Tables 1 and 2. Plates were incubated 2–5 days at (32.5 ± 2.5) °C and (22.5 ± 2.5) °C for bacteria and yeast and molds cultivation respectively.

For purposes of this study there were used several groups of non-sterile pharmaceutical products, namely: active substances for pharmaceutical use and different types of dosage forms (tablets, liquid solutions, syrups, aerosol and creams). Besides, some herbal medicinal products for oral use were examined.

Table 1
Formulation of nutrient media for bacteria growing.

Formula	g/l
Pancreatic fish protein hydrolysate	15.0
Pancreatic casein peptone	10.0
Yeast extract	2.0
Sodium chloride	3.5
D-glucose	1.0
Agar	10.0
pH 7.4 ± 0.2	

Table 2
Formulation of nutrient media for fungi growing.

Formula	g/l
Pancreatic fish protein hydrolysate	10.0
Pancreatic casein peptone	10.0
Yeast extract	2.0
Sodium dihydrogen phosphate	2.0
D-Glucose	40.0
Agar	10.0
pH 6.0 ± 0.3	

2.3. Plate-count methods

Direct plating for microbial enumeration was performed by spread-plate, pour-plate, two-layer agar techniques and its modification which differed from pour-plate method that 7–10 ml of media was used.

2.4. Inoculum standardization methods

The preparation of microbial suspensions was performed by using the international reference preparation of opacity and the DensiCHEK Plus instrument, which provides values in McFarland units, proportional to microorganism concentrations. Semi-quantitative and quantitative reference cultures such as Quanti-Cult^{PLUS} (Remel, USA), BioBall (Biomerieux, France) were also used.

Laboratory-specific uncertainty of counting was estimated. Several technicians were involved in microbiological analyses of the same samples independently.

The uncertainty of dilution factor (DF) was calculated for the case of the cultures suspensions preparation by serial decimal dilutions included 7 steps. Computation was based on the pipette certificate of analysis data according to G. G. Meynell procedure (Meynell and Meynell, 1970). Volumetric accuracy was 2% in each dilution step, being, 9.8 instead of 10, the real degree of dilution in 7 steps was assumed as $9.8^{-7} = 8.7 \times 10^{-6}$. The total error therefore was $(10^7 - 8.7 \times 10^6)/10^7 = 0.13$

2.5. Statistical analysis

Microbiological data do not normally conform to a “normal” distribution, and usually require mathematical transformation prior to statistical analysis (AOAC International, 2006). Bacterial counts often are characterized as having a skewed distribution. Therefore all microbiological data used in this study were converted to log values to achieve approximately normal distribution of the counts, before doing any statistical analysis (Technical Guide, 2008).

Statistical analysis was performed using Minitab 16.2.4. software, according to Paulson (2008). Comparisons were determined according to one way analysis of variance (ANOVA), based on the null hypothesis of equal means in each group. The Fisher criteria (F_c) was calculated and compared to table value (F_t). The level of statistical significance (α) was set at 0.05, 0.01 and 0.001. Probability value (p) was calculated. It was referred to statistically significant difference as $p < 0.05$ and statistically highly significant difference as $p < 0.001$. When a significant difference was detected, Tukey’s pair-wise comparisons were performed using the same software.

Considering all the elements of uncertainty (w_1 to w_n), the combined relative uncertainty of the test results (w_y) was estimated from the Eq. (1). Maximum relative standard deviation for each factor (w_i) was estimated based on the analysis of large number of observations (N) divided into several groups (factor