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Use of Total Error concept in the validation of viral activity in cell cultures☆

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ABSTRACT

Due to the high variability inherent of experimental recipients, validating biological methods is often a complex exercise, and following ICH Q2R1 recommendations is not always feasible and/or meaningful. Linking systematic error and random error to obtain a unique criterion, as defined in ISO guideline, could be of interest to capture the total variability in biological assays. In this paper, the use of Total Error concept in the validation of biological assays was for the first time investigated and compared to a conventional interpretation of the ICH guideline. Both decision methodologies concluded that the assay was valid from 2.13 to 5.83 $\log_{10}(CCID_{50}/ml)$. However, only the Total Error approach using accuracy profile as decision tool allowed to guarantee that accurate and reliable results will be obtained during the future routine application of the assay. In addition, the risk to obtain out of acceptance limits results was estimated using this approach and was found out to be at the most 3.1% irrespective of the concentration level, thus demonstrating the reliability of the biological assay.

1. Introduction

Biological assays differ from analytical methods in the way they are performed on living recipients such as cells, micro-organisms, animals, and plants. Each experimental unit has an individuality which confers characteristics differing from a unit to the other. As a result, variability sometimes important is observed. In addition to this variability inherent of experimental units, one has to consider all other sources of variation: unpredictable or systematic factors not under control (such as reagent lot effect, temperature effect, drift over time, specific interferences).

In the pharmaceutical industry, analytical validations are carried out according to the ICH guideline [1]. However, in the introduction of the guideline part II, it is stated that "*due to their complex nature, analytical procedures for biological and biotechnological products in some cases may be approached differently than in this document*". In sanofi pasteur, according to external and internal regulations, we try to keep as close as possible to ICH recommendations in validating biological assays, but in some cases it is not feasible because of the biological matrix (vaccines, sera) and/or the assay or both [2].

Among the characteristics of validation listed in the ICH guideline [1], two of them are of importance for quantitative assays: accuracy and precision. One common interpretation of this guideline is to evaluate separately the systematic error and the random error of the assay, which is then supposed to fulfil the evaluation of the accuracy and precision criterion, respectively. However, due to the confusion included in this ICH guideline as stated for example in [3], this approach mixes systematic error evaluation, represented by the trueness criterion, with Total Error evaluation represented by the accuracy criterion. Indeed, ISO guideline [4] provides an adequate definition for the determination of accuracy of quantitative methods, defined as the sum of trueness (ICH-Part II interpretation of Accuracy) and precision. This approach, also called "Total Error Concept", is being spread in the past few years for analytical methods validation and transfer [3,5–10]. Another complexity for validating biological assays is the evaluation of trueness and thus accuracy. Indeed, trueness assessment will depend on the availability of an international reference sample (provided for e.g. by World Health Organization - WHO or by the European Pharmacoepia), or of an internal reference sample or also on the existence of a reference assay. If none of these elements are available trueness and accuracy evaluation is not possible and only the evaluation of the assay precision will be achievable.

In this paper, we present for the first time the use of Total Error Concept in the validation of viral activity in cell cultures, as an example of quantitative biological assay. A comparison with one conventional interpretation of the ICH guideline, is also included in order to evaluate the interest of such approach in biological field.

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The intended use of this developed biological assay is to provide calibrated or reference samples of viral suspensions that will be used as positive controls for the methods used to detect the Avian Leukosis Virus (ALV) during intermediate production steps of viral vaccines produced on chicken cells.

2. Experimental

2.1. Analytical method

The analytical method is an in-vitro assay for the determination of virus concentration in cells: viral activity of Avian Leukosis Virus in chicken fibroblast cell line (DF-1) in microplates.

A cell suspension of the DF-1 line is distributed in 96-well microplates and incubated at 36 °C for 48 h, then inoculated with the viral suspension serially diluted. The microplates are incubated at 36 °C for 11–12 days then fixed with acetone.

The viral protein P27 common to avian retroviruses is detected by a specific antibody (rabbit antibody anti-P27, Charles River SPAFAS, Wilmington, MA). A secondary antibody (goat anti-rabbit IgG antibody, Clinisciences, Montrouge, France) linked to alkaline phosphatase is used as detector antibody, followed by addition of BCIP/NBT alkaline phosphatase substrate (Sigma-Fast, Sigma–Aldrich, Saint-Quentin Fallavier, France). A colour reaction will develop in the presence of the virus.

Enumeration of wells showing a black coloration (positive wells) allows calculating the infectious titre expressed in 50% infectious dose in logarithmic cell cultures per millilitre ($\log CCID_{50}$/ml), using the least squares method.

2.2. Validation design

The validation study was conducted using an in house reference viral suspension. The infectious titer of the undiluted reference viral suspension used for the validation study was established at 5.83 $\log_{10}(CCID_{50}$/ml). The four concentration levels were prepared by diluting the reference viral suspension to the 1:10, 1:100, 1:1000, 1:5000.

Since the method is a quantitative assay, characteristics assessed in the validation study were specificity, linearity, accuracy and precision. For the purpose of this article, only accuracy and precision will be presented.

For assessing accuracy, four concentration levels were prepared from the undiluted viral suspension, in order to cover the range of routine use. The four concentration levels were tested in three independent series (three different days – two operators).

For assessing precision, assays were carried out on the undiluted viral suspension. Three independent series were performed under conditions of intermediate precision: two operators –three different days. Within each series, six independent repetitions were performed under conditions ensuring repeatability: same operator - 1 day. Table 1 summarizes the validation design used.

2.3. Acceptance criteria

In addition to general acceptance criteria of GMP compliance such as equipment qualification and analysts training, specific acceptance criteria were defined for each validation characteristic.

For accuracy and precision, these specific acceptance criteria were the following:

- ICH-part II interpretation of accuracy: bias (difference between observed titre expressed in $\mathrm{Log}_{10}(CCID_{50}$/ml) and theoretical titre expressed in $\mathrm{Log}_{10}(CCID_{50}$/ml)) across the validation range should be included in the interval [-0.2 $\log_{10}(CCID_{50}$/ml); +0.2 $\log_{10}(CCID_{50}$/ml)].
- Precision: confidence interval of intermediate precision should not be greater than ± 0.5 $\log_{10}(CCID_{50}$/ml).

3. Results and discussion

3.1. Analytical results

The experimental results expressed in $\log_{10}(CCID_{50}$/ml) are shown in Table 1. They were obtained from two operators and during three days. Standard deviations and means are also given in Table 1.

3.2. Validation results

3.2.1. ICH conventional approach

3.2.1.1. ICH-Part II interpretation of accuracy. Bias (in $\log_{10}(CCID_{50}$/ml)) were calculated as the difference between the observed result and the expected one in Table 1. Bias values across the validation range were included in the interval [-0.2 $\log_{10}(CCID_{50}$/ml); +0.2 $\log_{10}(CCID_{50}$/ml)]. Homogeneity of all the results was verified by mean of an analysis of variance at $\alpha = 0.05$. Mean bias and 95% confidence limits were deduced:

$$\text{Mean bias} = -0.05 \log_{10}(CCID_{50}/\text{ml}) \ [-0.11; 0.00].$$

The assay is thus considered as accurate over the whole concentration range studied.

3.2.1.2. Precision. Statistical parameters were calculated from Table 1. They are presented in Table 2.

The 95% confidence interval calculated for one run and three repetitions, that is the design that will be applied in routine, met the acceptance criterion fixed at ± 0.5 $\log_{10}(CCID_{50}$/ml). The assay precision is thus acceptable.

3.2.2. Total error approach

An original approach to assess the validity of analytical methods is based on the concept of total error, by means of tolerance intervals [5–7,11]. Total error is the simultaneous combination of systematic and random errors [4–7]. The total error approach fulfils completely the validation requirements expressed in the ICH [1] or FDA [12] guidelines [3]. Moreover, this approach evaluates the validity of the method by examining the reliability of individual results obtained by the assay [9]. It provides an efficient and predictive decision tool: the accuracy profile [5–9]. The statistical methodology behind the accuracy profile decision tool relies on tolerance (or prediction) intervals [13,14].

Using the results of Table 1, it is possible to build an accuracy profile. Indeed, the true values of analyte present in the samples used are known and thus systematic errors evaluation is possible to add to the variability evaluation. All the validation results obtained from these data using the Total Error approach are included in Table 3. The acceptance limits were settled at $\pm 30\%$ of the reference samples concentration values expressed in $\log_{10}(CCID_{50}$/ml). The coverage of the tolerance intervals was set at the 95% level. As shown in Table 3, the maximum bias of the assay was observed for the 2.83 $\log_{10}(CCID_{50}$/ml) concentration level and did not exceed -0.10 $\log_{10}(CCID_{50}$/ml). The maximum intermediate precision standard deviation of the assay was also observed at this concentration level and reached 0.13 $\log_{10}(CCID_{50}$/ml). Finally, the accuracy of the biological assay, measured by the 95% β-expectation tolerance interval did not exceed the acceptance limits irrespective of the concentration level as indicated in Table 3. Fig. 1 illustrates this with the accuracy profile obtained for the validation of the ALV viral activity in chicken fibroblast cells culture assay. Fig. 1 and Table 3 show that the assay is valid over the whole concentration range studied. Fur-

Table 1
Experimental design used for the biological assay validation and analytical results obtained.

	Measurement	Dilution	Operator	Day	Run or Series	Observed result ($log_{10}CCID_{50}$/ml)	Mean ($log_{10}CCID_{50}$/ml)	Standard deviation ($log_{10}CCID_{50}$/ml)	Expected result ($log_{10}CCID_{50}$/ml)	Bias ($log_{10}CCID_{50}$/ml)
	1	1/5000	1	1	1	1.98			2.13	−0.15
	2	1/5000	2	2	2	2.12	2.09	0.10	2.13	−0.01
	3	1/5000	1	3	3	2.17			2.13	0.04
	4	1/1000	1	1	1	2.64			2.83	−0.19
	5	1/1000	2	2	2	2.88	2.73	0.13	2.83	0.05
	6	1/1000	1	3	3	2.68			2.83	−0.15
Accuracy	7	1/100	1	1	1	3.75			3.83	−0.08
	8	1/100	2	2	2	3.87	3.82	0.06	3.83	0.04
	9	1/100	1	3	3	3.85			3.83	0.02
	10	1/10	1	1	1	4.73			4.83	−0.10
	11	1/10	2	2	2	4.73	4.76	0.06	4.83	−0.10
	12	1/10	1	3	3	4.83			4.83	0.00
	13	1/1	1	1	1	5.97			5.83	0.14
	14	1/1	1	1	1	5.79			5.83	−0.04
	15	1/1	1	1	1	6.02	5.89	0.13	5.83	0.19
	16	1/1	1	1	1	5.74			5.83	−0.09
	17	1/1	1	1	1	5.79			5.83	−0.04
	18	1/1	1	1	1	6.01			5.83	0.18
	19	1/1	2	2	2	5.96			5.83	0.13
	20	1/1	2	2	2	5.81			5.83	−0.02
	21	1/1	2	2	2	5.83	5.87	]0.06	5.83	0.00
Precision	22	1/1	2	2	2	5.90			5.83	0.07
	23	1/1	2	2	2	5.85			5.83	0.02
	24	1/1	2	2	2	5.84			5.83	0.01
	25	1/1	1	3	3	5.82			5.83	−0.01
	26	1/1	1	3	3	5.65			5.83	−0.18
	27	1/1	1	3	3	5.74	5.73	0.06	5.83	−0.09
	28	1/1	1	3	3	5.74			5.83	−0.09
	29	1/1	1	3	3	5.71			5.83	−0.12
	30	1/1	1	3	3	5.74			5.83	−0.09

Table 2
Statistical parameters obtained using the classical interpretation of the ICHQ2 validation guideline [1].

Overall mean	5.83 $log_{10}(CCID_{50}/ml)$
Variance of repeatability	0.0074 with 15 df
Standard deviation of repeatability	0.09
Variance inter-series	0.0057 with 2 df
Variance of intermediate precision	0.0130 with 17 df
Standard deviation of intermediate precision	0.11
95% confidence interval of intermediate precision (for one run and three repetitions in routine)	±0.19 $log_{10}(CCID_{50}/ml)$

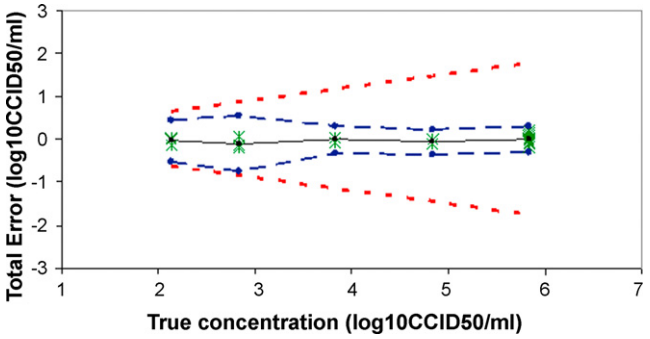

Fig. 1. Accuracy profile obtained for the validation of the viral activity method. The plain line is the bias, dashed lines are the β-expectation tolerance limit (β = 95%) and dotted lines represent the acceptance limit (±30% around the true value). The stars represent the relative back-calculated concentrations of the validation standards and are plotted according to their targeted concentration.

thermore, Fig. 1 shows that the lower limit of quantification is 2.13 $log_{10}(CCID_{50}/ml)$. Indeed, it is the smallest concentration tested for which the assay is able to provide accurate results. Therefore, at this concentration level and up to the highest one there are enough guarantees that, on average, at least 95% of future results will be within the ±30% acceptance limits.

Fig. 1 and Table 3 also indicate that the tolerance interval width is increasing when the concentration level decreases. This is due to the relatively poor experimental design for the first four concentration levels where only three series of one replicates were analyzed by comparison to the highest concentration level (5.83 $log_{10}(CCID_{50}/ml)$) where three series of six replicates were analyzed. This low amount of information increases the uncertainty of

Table 3
Validation results according to the Total Error approach.

Concentration level ($log_{10}CCID_{50}$/ml)	Trueness: mean bias ($log_{10}CCID_{50}$/ml)	Precision: intermediate precision S.D. ($log_{10}CCID_{50}$/ml)	Accuracy: tolerance limits ($log_{10}CCID_{50}$/ml)
5.83	0.00	0.11	[−0.30; 0.30]
4.83	−0.07	0.06	[−0.35; 0.22]
3.83	−0.01	0.06	[−0.33; 0.31]
2.83	−0.10	0.13	[−0.74; 0.54]
2.13	−0.04	0.10	[−0.53; 0.45]

S.D.: standard deviation.

Table 4
Estimates of the measurement uncertainties related to ALV activity at each concentration level investigated in validation.

Concentration log10(CCID50/ml))	Uncertainty of the bias log10(CCID50/ml))	Uncertainty log10(CCID50/ml))	Expended uncertainty log10(CCID50/ml))	Relative expended uncertainty (%)
5.83	0.048	0.124	0.248	4.2
4.83	0.033	0.067	0.133	2.8
3.83	0.037	0.074	0.148	3.9
2.83	0.074	0.148	0.297	10.5
2.13	0.057	0.114	0.227	10.7

the estimated bias and standard deviation and thus increases the width of the tolerance interval. Nonetheless, the method is shown perfectly valid and it provides enough guarantees concerning the quality of the results it will provide during its routine application.

Furthermore, the risk to obtain effective results out of acceptance limits due to the biological assay can be computed. The maximum risk tolerated was set at 5%. In other words, this means that it is expected that, in routine analysis, at most 5 sample measurements out of 100 will fall outside the ±30% acceptance limits. Fig. 2 shows the risk profile constructed by concentration level from the validation standards. The effective risk was about 3.1% for the two smallest concentration levels, and is less than 0.5% for the three other levels. Using this risk profile the analyst can see how far the biological assay is reliable for its intended use, therefore giving him a new tool to evaluate the reliability of its assay. The risk linked to the use of the analytical procedure by the subsequent user (the release laboratory) is known as required in the ICH Q9 document or by the Process Analytical Technology initiative of the FDA [15,16].

Validation is the first step to demonstrate results reliability, but is not enough if one aims at interpreting and comparing results correctly. Furthermore due to the intended use of the biological assay, uncertainty of measurements must therefore be evaluated to ensure this. One major advantage of the applied validation methodology is that it can, without any additional experiments, give estimation of uncertainty of measurements [17]. On this basis, several estimates of uncertainty were computed using the validation results and are presented in Table 4. The expanded uncertainty was computed using a coverage factor of $k = 2$ [18–20], representing an interval around the results where the unknown true value can be observed with a confidence level of 95%. As shown in Table 4, the relative expanded uncertainty for ALV concentration did not exceed 11% irrespective of the concentration levels. In other words and as long as it was demonstrated in validation that the assay is unbiased, this means that with a confidence level of 95% the unknown true value is situated at maximum ±11% around the measured result. Thus, when the assay will provide a result for an analyzed sample during routine application, the uncertainty of this result will be of maximum ±11%.

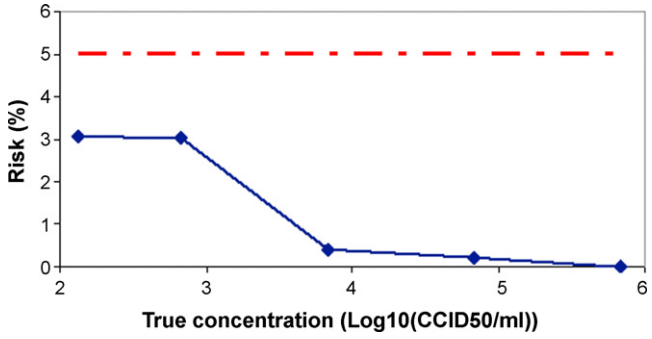

Fig. 2. Risk profile for the ALV viral activity method (continuous line) obtained by concentration level. The maximum tolerated risk is set at 5% (dotted and dashed line).

3.2.3. Discussion

The ICH document [1] aims at harmonizing analytical methods validation methodologies by providing minimum recommendations on experiments to perform together with the required data. However, this document is lacking recommendations on how to decide about the validity of analytical methods, leading thus to different approaches which do not always fulfil the final objective of the validation [9]. This objective is to ensure that the method under investigation will provide accurate and thus reliable results during its day-to-day application in routine analyses [5]. As shown in this paper, the ICH conventional approach leads to the conclusion that the method is valid over the range 2.13–5.83 $\log_{10}(CCID_{50}/ml)$. However, this approach only looks separately on the method bias (trueness) and variability (precision) and not on the reliability of the individual results. Indeed, the main aim of any quantitative method is to generate accurate results in order to make reliable subsequent decisions such as the batch release of pharmaceutical products. To circumvent this inconvenient and in order to answer adequately the objective of methods validation, the Total Error approach has been successfully applied. Indeed, this approach guarantees the reliability of the results that will be obtained by the methods; furthermore, the risk to obtain results out of acceptance limits is known before starting the day-to-day application of the method. In our example this risk is at maximum of about 3%. The conventional approach cannot provide such knowledge of the risks linked to the data generated by the analytical methods.

4. Conclusion

The objective of validation of an analytical method is "*to demonstrate that it is suitable for its intended purpose*" as stated in the ICH guideline. Beyond this fundamental objective, the expectation of laboratories and regulatory authorities is a guarantee that results obtained in routine will be as close as possible to the "true value". This implies in particular for quantitative assays a reduced bias and a small dispersion of results.

The approach described in the ICH guideline and its classical implementation in laboratories does not allow linking the two characteristics to obtain a unique criterion to decide on the capacity of the method to quantify. On the other hand, the Total Error approach using accuracy profiles allows to combine simultaneously systematic and random errors allowing to evaluate the reliability of the results that will be obtained by the assay during routine application. This methodology allows to make a decision about the validity of the assay knowing the potential risk of having inaccurate results in routine [9]. It answers adequately the objective of methods validation. Indeed, the risk to obtain out of acceptance limits results is known. In this paper, the application of Total Error approach was applied successfully for the first time to the validation of a biological assay. It has been shown to provide an efficient and potentially universal decision tool to assess biological assays validity.

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