⟨1223.1⟩ VALIDATION OF ALTERNATIVE METHODS TO ANTIBIOTIC MICROBIAL ASSAYS

INTRODUCTION

Microbiological assay methods have traditionally been used to quantify the potency, or antimicrobial activity, of antibiotics. These microbiological procedures were historically used to certify antibiotics on a lot-by-lot basis to ensure sufficient activity. Before 1998, monographs for several approved antibiotics were published in FDA's Code of Federal Regulations, 21 CFR. These regulatory antibiotic assay procedures were later published in *USP* as the official referee methods to determine the potency of antibiotics. The details of the microbial assay procedures for individual antibiotics, including the challenge organisms and test parameters, are described in general chapter *Antibiotics—Microbial Assays* ⟨81⟩. Microbial assays provide a direct measure of the effectiveness of the antibiotic against a reference microorganism. Although these microbiological methods have continued to serve as the official compendial referee methods since their publication in *USP*, many manufacturers have replaced these microbiological analyses with high-performance liquid chromatography (HPLC) methods.

Although the microbiological methods provide direct proof of antimicrobial effectiveness and can integrate all moieties that contribute to antimicrobial effects in a formulation, these methods are less precise, more complex to perform, and slower to complete than alternative methods such as HPLC. Microbial assays also have limited selectivity and are not appropriate for evaluating organic impurities. The specific skill sets required to perform the microbiological antibiotic assays, their unique equipment requirements, and their comparative complexity deter many stakeholders from using these methods.

There are numerous reasons for replacing the microbiological antibiotic assays with chemical assays that use purity or content as surrogates for the measurement of biological activity. The advantages of chemical-based analytical methods have been described previously for simple, single-component antibiotics, as well as complex, multi-component antibiotics (*1*). Physicochemical procedures, such as HPLC, allow for simpler preparation and rapid data acquisition with improved precision, accuracy, selectivity, and specificity. HPLC methods can be used effectively for both potency assignment and organic impurity testing. Additionally, because modern instruments and the expertise to use such equipment are widely available, the conversion to alternative methods may be economically advantageous.

This general chapter provides points to consider for manufacturers who want to use physicochemical alternatives instead of the microbial assay methods described in ⟨81⟩. Given the widespread use of HPLC as an alternative to microbial assay methods, this chapter focuses on HPLC methods. However, the principles set forth in this chapter are applicable to any alternative physicochemical procedure.

GENERAL CONSIDERATIONS FOR ALTERNATIVE METHOD DEVELOPMENT

Multiple important factors need to be considered when replacing microbial methods with HPLC or other chemical techniques; this is because of the specific characteristics of antibiotics.

1. It is essential to know whether the antimicrobial activity manifested by a preparation results from a single, active ingredient or arises from multiple, often related, moieties. Where technically possible, the analyst should determine the contribution of major moieties to antibiotic effectiveness. For the purposes of this chapter, major moieties are defined as those that contribute more than 1% of the antibiotic potency. Where multiple moieties contribute to antibiotic activity, the chemical assay should be able to resolve all major moiety peaks in the formulation.
2. To the extent possible, the individual activity of process impurities and degradation products should be evaluated. This may not be necessary where evidence exists that each of the impurities and degradation products contributes less than 1% of the total antimicrobial activity of a preparation.
3. General chapter ⟨81⟩ is the referee standard in any procedure comparison. Therefore, the currently official USP procedure should be performed by the manufacturer (or under that manufacturer's direction) to establish reference assay values within the test range. It may be necessary to evaluate data using the guidelines in general chapter *Analysis of Biological Assays* ⟨1034⟩ when the test design is one described in that chapter. Manufacturers should establish appropriate limits for precision and accuracy of the microbial assay procedure based upon their product knowledge.
4. The candidate alternative HPLC or other chemical method should be fully validated according to general chapter *Validation of Compendial Procedures* ⟨1225⟩.
5. The value of any method comparison is dependent upon the precision and accuracy of the assay test results obtained from both methods. No statistical comparison should be undertaken if the assay data from either method does not meet predetermined acceptance criteria for method validation.
6. The guidelines in this chapter do not apply to already marketed products for which the manufacturer has already received regulatory approval to use an alternative method. In all cases, the applicability of an alternative method can only be determined through submission and review by the relevant regulatory authority.
7. Any alternative to a compendial procedure must be validated and proven to be equivalent to, or better than, the referee method (*2*). A stimuli article published in *PF 35(3)* [May–June 2009] discusses the "equivalent or better" approach to evaluating alternatives to compendial procedures (*3*). It is expected that such a comparison will use appropriate statistical analysis. Examples of the recommended types of statistical analyses are outlined in this chapter.

TECHNICAL CONSIDERATIONS

Simple and complex antibiotics are treated separately because there are significant differences in methods development and validation for these two categories of antibiotics.

- Simple antibiotics (such as tetracycline) are those for which all antimicrobial activity is contributed by a single moiety.
- Complex antibiotics (such as gentamicin) are those that have more than one active moiety.

Stability-indicating alternative procedures are recommended. Where impurities or degradation products contribute more than 1% of the antimicrobial activity of an antibiotic preparation, impurities and degradation products must be evaluated.

The microbial assay and the candidate alternative method should be performed as described in the appropriate general chapter, e.g., ⟨81⟩ for microbial assays and general chapter *Chromatography* ⟨621⟩ for HPLC procedures.

BRIDGING STUDIES

Bridging studies are used to compare the data obtained from candidate alternative procedures to the microbial assay data to determine whether the alternative procedure is an acceptable substitute.

Simple Antibiotics

1. Separate the active antibiotic moiety from impurities and degradation products. Compare the microbial activity of the main moiety, process impurities, and degradation products against the USP Reference Standard for the antibiotic. Process impurities and degradation products present at levels below 1% of antimicrobial activity may be disregarded. The candidate alternative method may resolve impurities that do not have antimicrobial activity. These will likely not factor into the bridging study.
2. Continue as described in this section only if the product has a single moiety conveying the antimicrobial activity.
3. Validate the alternative procedure using ⟨1225⟩. The alternative procedure must be specific, selective, and stability indicating.
4. Test a minimum of three separate lots of the drug substance and the relevant USP Reference Standard using the microbial assay procedure as well as the candidate alternative method.
5. Test a minimum of six replicate samples per lot using both the microbial assay and candidate alternative method. It may be necessary to increase the number of replicates based on the maximum allowed percentage difference (see *Data Evaluation*) and the standard deviation of the method (*4*). If possible, prepare stock standard and sample solutions and subdivide them for use with the microbial assay and candidate alternative assay procedures. This provides paired data that can be analyzed (see *Appendix 2*).
6. Apply appropriate outlier (see general chapters *Analytical Data—Interpretation and Treatment* ⟨1010⟩ and ⟨81⟩) and comparison tests (see *Data Evaluation*) to determine if the candidate alternative method and the microbial assay procedures yield equivalent results.
7. If there are statistical outliers with either procedure, perform the comparison by excluding the outlier values. If the bridging study criteria are met, repeat the bridging study with controls to prevent outliers. Justification for rejection of outliers must be provided in the bridging study report. The consistent appearance of outliers in the candidate alternative assay results may indicate that the procedure has inadequate controls. If this occurs, the bridging study is void and a different alternative procedure should be developed and validated.
8. If the bridging study fails, it may be necessary to include potency contributions from impurities that are below 1%.

Complex Antibiotics

1. Separate and purify each antimicrobial moiety of a complex antibiotic preparation, process impurities, and degradation products. Compare the antimicrobial activity of the main moiety, process impurities, and degradation products against the USP Reference Standard for the antibiotic. Establish values for relative microbial activity (*F*) for each moiety as compared with the USP Reference Standard. Active moieties, process impurities, and degradation products at levels below 1% of antimicrobial activity may be disregarded. The candidate alternative method may resolve impurities that do not have antimicrobial activity. These will likely not factor into the bridging study.
2. Evaluate a suitable number of production lots of the antibiotic to determine whether the composition of the complex antibiotic is consistent from one lot to another.
3. Validate the candidate alternative procedure using ⟨1225⟩. The procedure must be specific, selective, and stability indicating.
4. Test a minimum of three separate lots of the drug substance and the USP Reference Standard using the microbial assay method and the candidate alternative procedure.
5. Test a minimum of six replicate samples per lot using both the candidate alternative method and the microbial assay procedures. It may be necessary to increase the number of replicates based on the maximum allowed percentage difference (see *Data Evaluation*) and the standard deviation of the method (*4*). If possible, prepare stock standard and sample solutions and subdivide them for use with the microbial assay and candidate alternative assay procedures. This provides paired data that can be analyzed (see *Appendix 2*).
6. Use the relative microbial activity (*F*) to convert the percentage purity values for each moiety, then sum them to determine a combined potency value.

7. Apply appropriate outlier (see ⟨1010⟩ and ⟨81⟩) and comparison tests (see *Data Evaluation*) to determine whether the candidate alternative procedure and the microbial assay procedure yield equivalent results.
8. If there are statistical outliers with either procedure, perform a comparison by excluding the outlier values. If the bridging study criteria are met, repeat the bridging study using controls to prevent outliers. Justification for rejection of outliers must be provided in the bridging study report. The consistent appearance of outliers in the candidate alternative assay results may indicate that the procedure has inadequate controls. If this occurs, the bridging study is void and a different or modified alternative procedure should be developed and validated.
9. If the bridging study fails, it may be necessary to include potency contributions from impurities that make a contribution below 1%.

DATA EVALUATION

Described below are USP's recommendations for data evaluation. Alternative approaches such as Deming regression (*5*) and commercially available comparison tests may also be used. For an antibiotic with a comparatively narrow Assay range (80%–125% or narrower), follow *Step 1*. For an antibiotic with a wide Assay range (80%–125% or wider), follow *Steps 1* and *2*.

1. Demonstrate the equivalence of results at the targeted potency of 100% using a two one-sided test (TOST) to test for equivalence. TOST offers several advantages over the *t*-test, which looks for differences (*6*,*7*,*8*,*9*). This requires the laboratory to set a maximum allowed percentage difference (such as 3%, 4%, or 5%), denoted by *k* in the appendices. Use the formulas in *Appendix 1* for independent samples (different samples used for the two procedures) or *Appendix 2* for paired samples (a set of samples each of which is assayed by both procedures).
2. Compare the chapter microbial assay and candidate alternative assay procedures using paired samples that cover the full monograph range of activity values. Prepare a Bland–Altman (BA) plot (*Appendix 3*). There should be no evidence of an important trend and the BA 95% agreement limits should not extend outside a predetermined maximum difference established by the laboratory.

APPENDIX 1: TOST FORMULAS FOR INDEPENDENT SAMPLES

On the basis of the knowledge of the product, the laboratory must establish the maximum allowed percentage difference between the average result for the candidate alternative assay and the average for the microbial assay results for the alternative assay. A difference that meets the requirements indicates that the alternative method provides acceptable results in the specified compendial range as compared with the microbial assay. In statistical notation, what must be demonstrated is:

$$100\left|\frac{\mu_{HPLC}}{\mu_{Micro}}-1\right|<100k$$

(Eq. 1)

k = a small positive number such as 0.03 (for an allowed 3% difference)
μ_i = mean value for each procedure
i = alternative method (HPLC) or microbial assay (Micro)
These are the two values to be compared.
Rearranging, this becomes:

$$-k\mu_{Micro} < \mu_{HPLC} - \mu_{Micro} < k\mu_{Micro}$$ (Eq. 2)

The idea of TOST is to consider the two inequalities of *Equation 2* separately. That is, to demonstrate the hypothesis in *Equation 2* at the 5% level, one must demonstrate both of the inequalities in *Equation 3* at the 5% level:

$$\mu_{HPLC} - (1 + k)\mu_{Micro} < 0 \text{ and } \mu_{HPLC} - (1 - k)\mu_{Micro} > 0$$ (Eq. 3)

For a comparison of means, the TOST is equivalent to considering two-sided 90% confidence bounds. If the bounds satisfy the inequalities in *Equation 3*, then equivalence has been demonstrated. When the samples for the two assays under comparison are different ("independent samples"), determine the following upper (*U*) and lower (*L*) confidence bounds:

$$U = \overline{X}_{HPLC} - (1+k)\overline{X}_{Micro} + t_{0.05,df}\sqrt{S^2_{HPLC}/N_{HPLC} + (1+k)^2 S^2_{Micro}/N_{Micro}}$$

(Eq. 4)

$$L = \overline{X}_{HPLC} - (1-k)\overline{X}_{Micro} - t_{0.05,df}\sqrt{S^2_{HPLC}/N_{HPLC} + (1-k)^2 S^2_{Micro}/N_{Micro}}$$

(Eq. 5)

$\overline{X}_i$ = sample average
S_i = sample standard deviation
N = sample size
t = one-sided 5% value for a t-distribution
i = alternative method (HPLC) or microbial assay method (Micro)
For the number of degrees of freedom, use:

$$\frac{\left(\frac{s_{Micro}^2}{N_{Micro}}+\frac{s_{HPLC}^2}{N_{HPLC}}\right)^2}{\left[\left(\frac{1}{N_{Micro}-1}\right)\left(\frac{s_{Micro}^2}{N_{Micro}}\right)^2\right]+\left[\left(\frac{1}{N_{HPLC}-1}\right)\left(\frac{s_{HPLC}^2}{N_{HPLC}}\right)^2\right]}$$

(Eq. 6)

This is an approximation that assumes k is small. If using software that only allows for integer degrees of freedom (e.g., Excel), use linear interpolation to obtain the t-value. Conclude that the two procedures are equivalent (i.e., any difference on average is acceptably small) for the given lot if:

$$L > 0 \text{ and } U < 0 \quad \text{(Eq. 7)}$$

Example 1:

Microbial Assay Data	HPLC Assay Data
72.02	72.68
67.3	72.24
71.79	72.5
71.16	—
69.06	—
75.56	—
74.7	—
74.16	—
76.48	—

Following the above formulas with $k = 0.03$:

$$N_{HPLC} = 3;\ \overline{X}_{HPLC} = 72.5;\ S_{HPLC} = 0.221$$

$$N_{Micro} = 9;\ \overline{X}_{Micro} = 72.5;\ S_{Micro} = 3.045$$

Degrees of freedom = 8.247; interpolated t-value = 1.853

$L = 0.338$ and $U = -0.219$, so the 3% equivalence criterion is satisfied.

APPENDIX 2: TOST FORMULAS FOR PAIRED SAMPLES

When the samples for the two assays are the same, the data are considered "paired". The hypotheses are the same as those in *Appendix 1*. Because of the pairing of samples, the standard deviation calculations differ.

Determine the confidence bounds, U and L, as follows:

$$U = \overline{X}_{HPLC} - (1+k)\overline{X}_{Micro} + t_{0.05,df}\sqrt{S_U^2 / N}$$

(Eq. 8)

$$L = \overline{X}_{\text{HPLC}} - (1-k)\overline{X}_{\text{Micro}} - t_{0.05,df}\sqrt{S_L^2 / N}$$

(Eq. 9)

N = number of samples for each procedure
$df = N - 1$
S_L and S_U are calculated as follows:

$$S_U^2 = \frac{1}{N-1}\sum_{j=1}^{N}[X_{\text{HPLC},j} - (1+k)X_{\text{Micro},j}]^2$$

(Eq. 10)

$$S_L^2 = \frac{1}{N-1}\sum_{j=1}^{N}[X_{\text{HPLC},j} - (1-k)X_{\text{Micro},j}]^2$$

(Eq. 11)

Conclude that the two procedures are equivalent (i.e., any difference on average is acceptably small) for the given lot if:

$L > 0$ and $U < 0$ (Eq. 12)

Example 2:

Microbial Assay Data	HPLC Assay Data
1011	980.9
990	981.4
960	978.3
1000	974.3
970	966.7

Following the above formulas with $k = 0.03$:

$N = 5$; $\overline{X}_{\text{HPLC}} = 976.3$; $\overline{X}_{\text{Micro}} = 986.2$

$S_L = 18.749$; $S_U = 19.958$

$df = 4$; t-value = 2.132

$L = 1.830$ and $U = -20.438$, so the 3% equivalence criterion is satisfied.

APPENDIX 3: BLAND–ALTMAN PLOTS

Figure 1 shows the data plotted as HPLC (x-axis) vs. Micro (y-axis). In *Figure 2*, the x-axis represents the average response obtained using the alternative and microbial assay methods.

The y-axis represents the difference in responses measured with the alternative and microbial assay methods.

The bias for each data set is represented by the average difference.

95% limits of agreement are represented by bias ± 2S.

Data points outside the limits of agreement are considered outliers.

The Bland–Altman plot (*10*,*11*) should show data points scattered within the limits of agreement with no obvious pattern. If a pattern exists (e.g., proportional pattern or funneling outwards with increasing average), perform a log transformation. If performing a log transformation, take the antilog of results for analysis using the original scale of measurement.

Example 3:

Level	Microbial Assay Data	HPLC Assay Data
150	854	867

Level	Microbial Assay Data	HPLC Assay Data
150	862	893
150	871	880
150	845	906
150	836	854
120	678	704
120	699	722
120	688	739
120	705	686
120	671	669
90	530	541
90	536	554
90	522	528
90	510	515
90	515	502
60	366	343
60	363	361
60	348	334
60	352	370
60	357	352
30	197	185
30	187	172
30	192	176
30	189	180
30	195	167

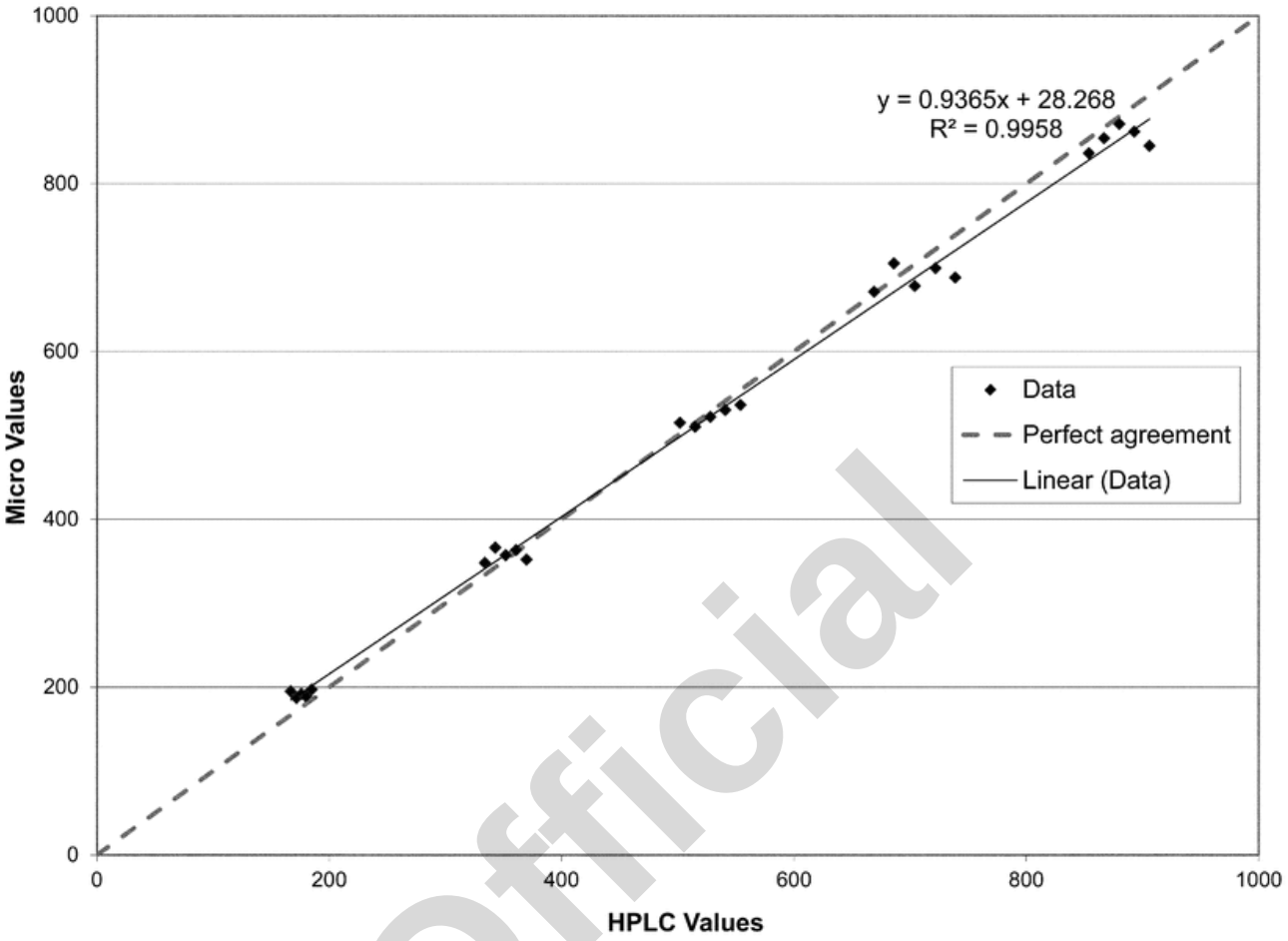

Figure 1. Example data plotted as Microbial assay data versus HPLC assay data.

Figure 1 shows the data plotted as Micro (*y*-axis) vs. HPLC (*x*-axis). This appears to be in good agreement. *Figure 2* shows what happens when the same data are instead plotted as recommended by Bland and Altman.

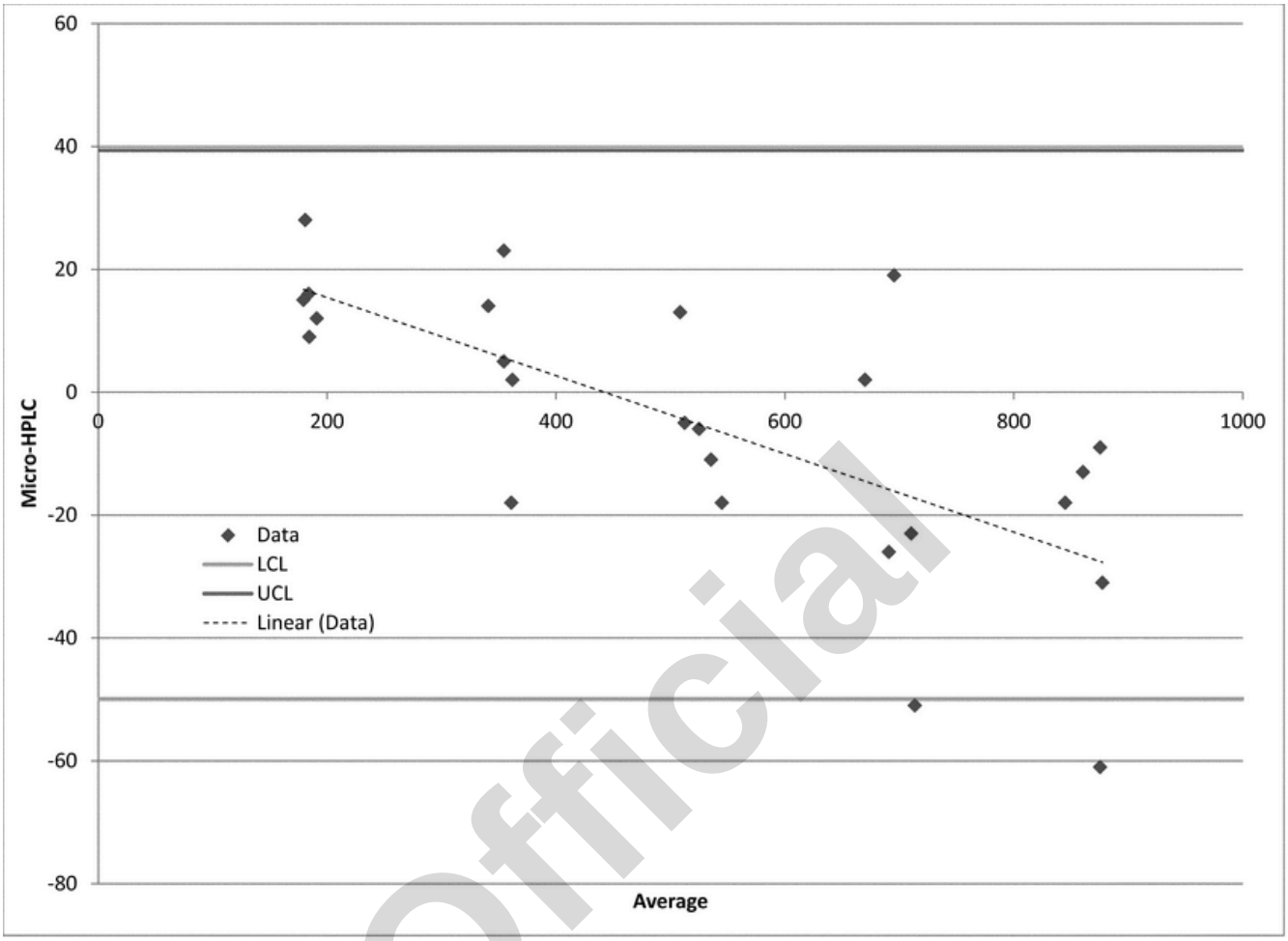

Figure 2. Example data in a Bland–Altman plot.

Calculate the bias, estimated by the mean difference (d) and standard deviation (SD) of the difference.

Mean difference = –5.28

SD of difference = 22.36

Most of the differences are expected to lie between d – 2SD and d + 2SD (if normally distributed, 95% will lie in these limits). If differences within d ± 2SD (limits of agreement) are not important, the two measurement methods can be used interchangeably.

LCL (d – 2SD) = –49.95 (Eq. 13)

UCL (d + 2SD) = 39.39 (Eq. 14)

The difference between results from the two procedures has a strong trend. This trend can be seen in *Figure 1*, but the Bland–Altman plot in *Figure 2* makes it clearer.

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