Research Article

A Sensitivity Analysis of the Modified Chi-square Ratio Statistic for Equivalence Testing of Aerodynamic Particle Size Distribution

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Abstract. Demonstration of equivalence in aerodynamic particle size distribution (APSD) is one key component for establishing bioequivalence of orally inhaled drug products. We previously proposed a modified version of the Chi-square ratio statistic (mCSRS) for APSD equivalence testing and demonstrated that the median of the distribution of the mCSRS (MmCSRS) is a robust metric when test (T) and reference (R) cascade impactor (CI) profiles are identical. Here, we systematically evaluate the behavior of the MmCSRS when T and R CI profiles differ from each other in their mean deposition and variability on a single and multiple sites. All CI profiles were generated by Monte-Carlo simulations based upon modified actual CI data. Twenty thousand sets of 30 T and 30 R CI profiles were simulated for each scenario, and the behavior of the MmCSRS was correlated to metrics that characterize the difference between T and R product in mean deposition and variability. The two key findings were, first, that the MmCSRS is more sensitive to difference between T and R CI profiles on high deposition sites, and second, that a cut-off value for APSD equivalence testing based on the MmCSRS needs to be scaled on the variability of the R product. The former is considered as beneficial for equivalence testing of CI profiles as it decreases the likelihood of failing identical CI profiles by chance, in part, due to increasing analytical variability associated with lower deposition sites. The latter is expected to be important for consistently being able to discriminate equivalent from inequivalent CI profiles.

KEY WORDS: aerodynamic particle size distribution; bioequivalence; cascade impactor; modified Chi-square ratio statistic; orally inhaled drug products.

INTRODUCTION

Demonstration of equivalence in aerodynamic particle size distribution (APSD) is one of several key components in the aggregate weight of scientific evidence approach that the FDA proposed for establishing bioequivalence (BE) of orally inhaled drug products (OIDPs) (1). APSD equivalence between a test (T) and reference (R) OIDP can be assessed by comparative analysis of multi-stage cascade impactor (CI; e.g., Andersen cascade impactor or next-generation impactor) data. A modified chi-square ratio statistic (mCSRS; Eq. 1)

was introduced as a potential metric for equivalence testing of APSD in the first part of this series of three articles (2).

$$\text{mCSRS}_{jk} = \frac{\sum_{i=1}^p \frac{(T_{ij} - \bar{R}_i)^2}{\bar{R}_i}}{\sum_{i=1}^p \frac{(R_{ik} - \bar{R}_i)^2}{\bar{R}_i}} \quad (1)$$

where p represents the number of deposition sites of the CI profile, T_{ij} and R_{ik} represent the normalized deposition (i.e., by the dividing the absolute deposition on the i th site by the total deposition on all sites under consideration) on the i th site of the j th CI profile ($j=1, \dots, n_T$) of the T sample and on the i th site of the k th CI profile ($k=1, \dots, n_R$) of the R sample, respectively. n_T and n_R represent the number of CI profile samples that were obtained from the T and R product, respectively, and $\bar{R}_i$ represents the sample mean on the i th site of all R CI profiles.

The mCSRS is a modified version of the CSRS that was proposed as a statistical method for equivalence testing of CI profiles in the June 1999 *Draft Guidance for Industry: Bioavailability and Bioequivalence Studies for Nasal Aerosols and Nasal Sprays for Local Action* (3). The previous study (2) evaluated the behavior of the distribution of the 900 mCSRSs of 30 T and 30 R CI profiles when the two products were identical (i.e., all 60 CI profiles originate from the same product). The key finding of this study was that the median of

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the distribution of 900 mCSRSs (MmCSRS) consistently yields one when T and R CI profiles are identical regardless of the shape and number of deposition sites of a CI profile. Hence, the MmCSRS is a potential test statistic for APSD equivalence testing and can be applied to deposition sites that may be considered more relevant to lung deposition. In contrast, it was reported that the “stability” of the test statistic for the CSRS was dependent on the shape and decreased with the number of CI deposition sites, and thus this metric could not be applied to a CI profile with a reduced number of deposition sites (4,5).

However, the application of the MmCSRS for the APSD equivalence testing warrants adequate understanding of the behavior of this metric when T and R CI profiles differ from each other. In this article, the behavior of the MmCSRS when T and R CI profiles differed were systemically evaluated in a series of simulations that varied in complexity with respect to both differences in mean deposition (single site vs. multiple site differences) and variability (variability of the T product is identical or differs from that of the R product).

METHODS

Monte-Carlo simulations were used to generate CI profiles that were, subsequently, used for evaluating the behavior of the MmCSRS. Within these simulations, CI profiles with defined mean depositions and variability were generated from a p -dimensional random vector X with p -dimensional population mean vector μ and $p \times p$ -dimensional population covariance matrix Σ , where p represents the number of deposition sites of the CI profile. The i th element ($i=1, \dots, p$) of X and μ represented the observed deposition and the population mean deposition on the i th site of the CI profile, respectively. The i th diagonal element of Σ represented the population variance on the i th site of the CI profile. The off-diagonal elements of Σ represented the population covariance between two sites of the CI profile. Furthermore, it was assumed that X follows a multivariate normal distribution (6,7). The “R” extension package “mvtnorm” (8) was used for random sample generation from the multivariate normal distribution. In the case that a negative deposition on a site was simulated, its value was set to 0.001 μg . A discussion on the selection of this value and the assumption of normally distributed CI data is given in the first paper of the series of three publications (2). In this paper, “observed” T and R CI profiles were based on simulated profiles defined by their respective population mean vectors and their respective population covariance matrices. Differences in mean deposition on a single or multiple site (s) between two CI profiles were achieved by altering the respective elements of μ accordingly. Variability adjustment (*i.e.*, when two CI profiles differed in their variability) without affecting the inter-site correlation (*i.e.*, the correlation between the deposition on different sites) was achieved by decomposition of the Σ as

$$\Sigma = D^{1/2} R D^{1/2} \quad (2)$$

where $D^{1/2}$ is a matrix whose i th diagonal element represents the population standard deviation of the i th site of the CI profile and R is the population (inter-site) correlation matrix.

Thus, the variability of the i th site was modified by changing the i th diagonal entry in $D^{1/2}$. CI profiles were simulated in units of mass deposition and, subsequently, normalized by dividing the mass on individual sites by the total mass of all deposition sites. It should be emphasized here that differences between T and R CI profiles always apply to absolute depositions (*i.e.*, μg scale) and that the mCSRS is always applied to normalized CI profiles throughout this articles. In the case of the single site differences, a difference in absolute deposition resulted in difference on all deposition sites after normalization (see below). In the case of multiple site differences, differences in absolute deposition were constructed such that the mass balance was maintained even prior to normalization (see below).

Evaluation of the Behavior of the MmCSRS When T and R CI Profiles Differ From Each Other On A Single-Deposition Site

For scenarios where the T and R CI profiles differed from each other in their mean deposition on a single site, the behavior of the MmCSRS was characterized for T and R CI profiles with the same variability as well as for T CI profiles that were more and less variable than the R CI profiles. μ and Σ of the R CI profiles were based upon a modified version of actual CI data, which comprised 11 deposition sites. Specifically, the R CI profile was supposed to represent a typical CI profile of OIDs, such as nebulizers, and allowed evaluation of the effect of the location (*e.g.*, low vs. high deposition sites) of single site changes on the MmCSRS. The mean depositions and standard deviations of all deposition sites are displayed in Fig. 1. The inter-site correlation structure is provided as supplemental material (Table S1).

Single Site Mean Difference—T and R CI Profiles with Identical Variability

T and R CI profiles differed from each other on a single site in their mean deposition by -90% , -80% , -70% , ..., -10% , 0% , 10% , ..., and 70% , 80% , or 90% . For instance, if the deposition on site 1 of the R CI profile is 5 μg and a 60%

Fig. 1. Reference CI profile that was used for the evaluation of the impact of differences between T and R CI profiles on a single-deposition site on the MmCSRS. For each of the 11 sites, the mean and standard deviation (in mg) of the deposition is displayed in the legend. Total deposition across all stages is 366.55 mg

mean difference was studied, then the deposition on site 1 of the T CI profile is changed to $1.6 \times 5 = 8 \mu\text{g}$. Subsequently, the deposition on all sites is normalized to % of the total deposition (see above) and, as a consequence, there will be a mean difference between T and R on all deposition sites (in % scale but not in μg scale). The above differences were applied to all of the 11 deposition sites, one site at a time. T and R CI profiles had the same variability. Thus, a total number of 209 (19×11) scenarios were generated in this part of the study.

Single Site Mean Difference—T and R CI Profiles with Different Variability

T and R CI profiles differed from each other on a single site in their mean deposition by 10% or 30%.¹ These differences were applied to the deposition sites 1, 7, 3, and 11 (Fig. 1) that represent high (approximately 53% of total deposition (*i.e.*, 194.51 mg of 366.55 mg), medium (11.7%), low (4.7%) and very low (0.36%) deposition sites, respectively. The effects of these differences on the MmCSRS were evaluated for R products with a high variability (variances increased by a factor of five compared to Table S1), medium variability (variances identical to Table S1), and low variability (variances decreased by a factor of five compared to Table S1). Furthermore, the variability of the T CI profiles was adjusted such that the T/R variability ratio was 0.1, 0.2, 0.5, 2, 5, or 10 on all sites. The T/R variability ratio was assumed to be the same on all deposition sites to keep the complexity of the results and their interpretation at a manageable level. However, the inter-site correlation structure (Table S1) was maintained. A total number of 168 ($2 \times 4 \times 3 \times 7$) scenarios were generated in this part of the study.

For each of these 377 scenarios (209 with identical+168 with different variability), 20,000 sets of 30 T and 30 R CI profiles were simulated and the MmCSRS was recorded for all of the 20,000 sets. The average of the 20,000 MmCSRS was then used for evaluating the effect of single site differences between T and R CI profiles on the behavior of the MmCSRS.

Since the T and R CI profiles were constructed by modification of the absolute deposition but the mCSRS is applied to normalized CI profiles (*see above*), the normalized squared difference reference scaled (NSDRS; Eq. 3)) was designed to correlate (*i.e.*, regression analysis) the observed differences in MmCSRS and the differences between T and R CI profiles (after normalization) between the 209 scenarios for which T and R CI profiles had the same variability.

$$\text{NSDRS} = \sum_{i=1}^p \frac{(T_i^{\text{Pop}} - R_i^{\text{Pop}})^2}{R_i^{\text{Pop}}} \quad (3)$$

where p is defined above and T_i^{Pop} and R_i^{Pop} represent the i th element of the normalized population mean vectors of the T and R CI profiles, respectively. The NSDRS is introduced here to provide a numerical description for the expected behavior of the MmCSRS when T and R CI profiles differ from each other on a single site (*i.e.*, a difference on a single site in absolute deposition will result in a difference on all sites after normalization; see above and “Discussion”).

¹ These two numbers were arbitrarily chosen but could possibly represent accepted differences for equivalence testing of CI profiles

Evaluation of the Behavior of the MmCSRS When T and R CI Profiles Differ From Each Other on Multiple Deposition Sites

Since the value of the MmCSRS, according to its computational form, is independent of the ordering of the deposition sites it was sufficient to systematically study rank-ordered CI profiles (*i.e.*, deposition sites were ordered according to their decreasing magnitude of normalized drug deposition). The CI profiles M1–10 (Fig. 2, presented as rank-ordered CI profile of different shapes) were used for assessment of the impact of changes on multiple deposition sites on the behavior of the MmCSRS. All of the ten CI profiles M1–10 consisted of eight deposition sites that could, for instance, represent the sites that are comprised in the definition of the impactor-sized mass (ISM)² for the Andersen CI (see “Discussion”). The mean vectors of CI profiles M1–10 were constructed as follows to cover a wide range of theoretically possible CI profiles (*i.e.*, from a uniform to extremely skewed distribution) and to facilitate the understanding of the complex analysis related to multiple-site changes. The deposition on all eight sites summed up to $100 \mu\text{g}$ for both T and R CI profiles. Furthermore, the following two restrictions were made in order to maintain the total deposition mass of $100 \mu\text{g}$ after differences on multiple deposition sites between T and R CI profiles were introduced. First, for the R CI profiles, the deposition on one site was equal to that of at least one other site. Second, the T CI profiles were constructed by letting the deposition on four sites be higher than those of the R CI profile and the deposition on the other four sites be lower than those of the R CI profile, while pairing two sites with identical depositions. Table 1 gives the mean vectors of two specific T and R CI profiles for better illustration of this procedure. In order to facilitate interpretation of the results, the variances of the CI profiles M1–10 were constructed such that all eight CI sites had identical coefficients of variation (CVs). The effect of different inter-site correlation structures on the behavior of the MmCSRS was studied by generating the CI profiles M1–10 with (*i.e.*, deposition on different sites are correlated with each other) and without (*i.e.*, deposition on different sites are independent of each other) inter-site correlation.

In detail, the following procedure was applied to the CI profiles M1–10 for constructing T and R CI profiles that differed from each other on multiple sites in their mean depositions and/or variability.

First, the inter-site correlation structure was selected between two possibilities. In particular, there was either no inter-site correlation or the inter-site correlation was based upon the covariance matrix (sites 4 – 11) that was used for the evaluation of the effect of single site changes on the MmCSRS (Table S1).³ The same inter-stage correlation structure was then applied to both T and R CI profiles. Second, the CVs were set on each of the eight deposition sites of the R CI profile to the same value of 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%. These values were chosen as they could possibly cover a range of realistically

² ISM is defined as the sum of the drug mass on all CI stages plus the filter, but excluding the initial stage because of its lack of a specified upper cutoff size limit (3).

³ It must be noted that the purpose of this was rather to create some correlation between the deposition sites than to model realistic inter-site correlation.

Fig. 2. Population mean deposition (in micrograms) of the CI profiles “M1”–“M10,” presented as rank-ordered CI profiles profile (*i.e.*, deposition sites were ordered according to their decreasing magnitude of normalized drug deposition)

observed CVs for CI profiles. It should be noted that the assumption of identical CVs on all deposition sites is not expected for real CI profiles but was mainly used here for systematic evaluation and understanding of the MmCSRS behavior (see “Discussion”). Third, the difference between the T and R CI profiles in their mean depositions was set to 5%, 10%, 15%, 20%, 25%, or 30% on all eight sites (*see above* and Table I). These values were chosen to cover a certain range of differences between CI profiles that could possibly be considered as equivalent. Fourth, the standard deviations on the depositions sites of the T CI profile were adjusted such that their CVs were half as large, the same as, or double as large as those of the R CI profile.

Hence, a total number of 3,240 ($10 \times 2 \times 9 \times 6 \times 3$) scenarios were evaluated. For each of the 3,240 scenarios, 20,000 sets of 30 T and 30 R CI profiles were simulated and the average of the MmCSRS across the 20,000 sets was recorded. The scenarios were then grouped according to their inter-stage correlation structure, the shape of the R profiles (*i.e.*, M1–10), and the difference in mean deposition between T and R CI profiles. For the 1,080 scenarios for which T and R CI profiles had the same

variability, the averages of the MmCSRS were then regressed against the respective squared inverse of the CV ($SqInCV$) separately for each of the groups (*see above*) to relate the change in MmCSRS to the variability of the R product. Subsequently, the estimated slopes and intercepts of this regression analysis were numerically compared with the normalized squared difference (NSD; Eq. 4) between T and R CI profiles to explore the influence of the inter-site correlation structure, the different shape of the CI profiles, and the difference in mean deposition on the MmCSRS.

$$NSD = \sum_{i=1}^p (T_i^{Pop} - R_i^{Pop})^2 \quad (4)$$

RESULTS

Evaluation of the Behavior of the MmCSRS When T and R CI Profiles Differ from Each Other on a Single Site

Single Site Mean Difference—T and R CI Profiles with Identical Variability

The behavior of the MmCSRS when T and R CI profiles differed from each other in their mean deposition on only a single site while both T and R CI profiles had the same variability is displayed in Fig. 3 for all of 209 scenarios under consideration.

Linear regression of the averages of the 20,000 MmCSRS against the NSDRS (Eq. 3), as a measure of differences between two CI profiles after normalization (see “Introduction”), displayed a perfect linear relationship (coefficient of determination (R^2) > 0.999) between the MmCSRS and the NSDRS (Fig. 4). This relationship was expected from the computational form of the mCSRS given that the mCSRS is applied to normalized CI profiles. A more detailed explanation of this relationship is given in the “Discussion.”

Single Site Mean Difference—T and R CI Profiles with Different Variability

The behavior of the MmCSRS when T and R CI profiles differed from each other in their mean deposition on a single site by 30% and had a different variability on all sites is shown in Fig. 5. The results for a 10% difference in mean deposition between T and R CI profiles are provided as

Table I. Illustration of Multiple Site Change Procedure for CI Profiles M1–10 as an Example Shown for the Profiles M1 and M10 (Fig. 2)

Profile/stage	1	2	3	4	5	6	7	8
M1–R	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
M1–T	13.75	11.25	13.75	11.25	13.75	11.25	13.75	11.25
Difference	10%	–10%	10%	–10%	10%	–10%	10%	–10%
M10–R	20	20	15	15	10	10	5	5
M10–T	24	16	18	12	12	8	6	4
Difference	20%	–20%	20%	–20%	20%	–20%	20%	–20%

Entries for the reference (R) and test (T) products are the site depositions in micrograms

Fig. 3. Analysis of the behavior of the MmCSRS. T and R CI profiles differed from each other on a single stage in their mean deposition by a certain percentage (x -axis). T and R CI profiles had identical covariance matrices. The effect of single site difference was evaluated on all 11 sites of the CI profile that is displayed in Fig. 1; *right panel* is a magnification of the *left panel*

supplemental material (Fig. S2). For all scenarios, the MmCSRS increased as the T/R variability ratio increased. However, for a constant T/R variability ratio, the MmCSRS was increased and decreased for a highly variable and less variable R product, respectively. Furthermore, a partial linear relationship⁴ between the MmCSRS and the T/R variability ratio was observed for a T/R variability ratio between 1/10 and 10.

Evaluation of the Behavior of the MmCSRS When T and R CI Profiles Differ from Each Other on Multiple Deposition Sites

The behavior of the MmCSRS when T and R CI profiles differ from each other on multiple sites in their mean deposition and/or variability is displayed in Figs. 6 and 7 for the CI profiles M2 (no inter-site correlation) and M5 (with inter-site correlation), respectively. Plots for all ten CI profiles M1–10 (with and without inter-site correlation), which show similar behavior, are provided as supplemental material (Figs. S3–S20).

For the scenarios where T and R CI profiles had the same variability, linear regression of the average of the 20,000 MmCSRSs against the SqInCV separately for each group (grouping was based upon the inter-site correlation structure, the shape of the R profiles (i.e., M1–10), and the difference between T and R CI profiles; *see above*) yielded a perfect linear relationship ($R^2 > 0.999$) (Table II). The estimated slope and intercept parameters from the simple linear regression analysis are given in Table II. All estimated intercept parameters were between 0.8 and 1.3 and negatively correlated with the NSD (Table II; Fig. 8, top-left panel; Pearson product-moment correlation coefficient (r) = -0.566). Furthermore, the estimated intercept parameters seem to be independent of the inter-site correlation structure (Fig. 8, top-

left and bottom-left panel). The estimated slope parameters increased with increasing NSD (Table II; Fig. 8: top-right panel) while the two metrics were highly correlated with each other ($r = 0.941$). The estimated slope parameters of the scenarios with and without inter-site correlation are linear functions of each other ($R^2 = 0.97$; Fig. 8, top-right panel).

DISCUSSION

In a previous publication (2), we introduced the MmCSRS and characterized its behavior when T and R profiles did not differ from each other. Under these conditions, the MmCSRS was found to be one, regardless of the shape and the number of deposition sites of the CI profiles. This behavior was promising and indicated that the MmCSRS is a robust metric for APSD equivalence testing and potentially useful for determining whether T and R CI profiles are equivalent.

As a next step, it was of interest to characterize the behavior of the MmCSRS (e.g., the expected increase of the MmCSRS) under conditions where T and R CI profiles are not identical. This paper evaluated the behavior of the

Fig. 4. Analysis of behavior of MmCSRS for single site difference between T and R CI profiles with identical variability. Simple linear regression of average of MmCSRS ($n = 20,000$) vs. NSDRS (Eq. 3) was used for quantification of the difference between T and R CI profiles after normalization. RSQ coefficient of determination

⁴ Further evaluation of the behavior of the MmCSRS for larger T/R variance ratios than 10 was performed and showed that the MmCSRS increased disproportionately with increasing T/R variance ratio. Specifically, the MmCSRS seemed to approach a certain maximum. However, since cases for which the variability of the T CI profile are more than 10 fold larger than that of the R CI profile are very unrealistic and, thus, were not considered in this article.

Fig. 5. Analysis of the behavior of the MmCSRS (y-axis, average of 20,000 replicates) when T and R CI profiles differed from each other in their mean deposition on a single site by 30% and in their variability on all sites by a factor of 0.1 through 10 (x-axis); *site 1*, high-deposition site; *site 3*, low-deposition site; *site 7*, medium-deposition site; *site 11*, very low-deposition site; *circles*, highly variable R product (variances increased by a factor of 5 compared with Table S1); *triangles*, normally variable R product (variances identical to Table S1); *plus sign*, less variable R product (variances decreased by a factor of 5 compared with Table S1)

MmCSRS under such conditions using a systematic approach of increasing complexity. First, simulations evaluated the behavior of MmCSRS when T and R CI profiles differed only on a single site, while the variability was identical; followed by simulations that also allowed differences in variability between T and R products. Subsequently, T and R CI profiles differed on multiple deposition sites from each other. However, the overall deposited mass on all sites under consideration was maintained constant (see above), as this would be the prerequisite for a T product before performing an APSD equivalence test based upon the MmCSRS. Such a prerequisite will be further discussed in a forthcoming article (part III). Maintaining mass balance between T and R CI profiles was not possible for single site difference (see above). For the multiple-site differences scenarios, T and R CI profiles either had the same or a different variability. Knowing the behavior of MmCSRS under such conditions was important, especially when the variability of the R product would affect MmCSRS. Under such conditions, a reference variance scaling approach might have to be employed for establishing critical values for determining APSD equivalence of the T to R OIDP.

Single Site Mean Differences

In this part of the study, a total number of 11 deposition sites (8 of which may be thought to be more relevant for pulmonary deposition, *e.g.*, definition of ISM for Andersen

CI) were incorporated to study the behavior of the MmCSRS over wider range of sites with a distinct difference in drug deposition across the investigated sites (deposition on non ISM sites (*e.g.*, pre-separator) is generally higher). When T and R CI profiles differed from each other in their mean deposition on a single site, while variability associated with both profiles was identical, the MmCSRS increased as the difference between T and R CI profiles increased (Fig. 3). This was true for all sites, independent on what site showed the difference. However, this increase was not symmetric around 1 as, for example, a 70% increase or a 70% decrease in a specific site deposition resulted in different MmCSRS values.

The extent of change in MmCSRS was also dependent on how much drug was deposited on a given site (Fig. 3). As an example, a difference in mean deposition of 50% between T and R CI profiles would result in an MmCSRS of more than 15 for the high deposition site 1, compared with an MmCSRS of 1 if T and R were identical (Fig. 3). If a 50% difference is observed for one of the low deposition stages (*e.g.*, site 11 in Fig. 3), MmCSRS hardly changes at all (very close to 1).

Thus, the increase in MmCSRS is more pronounced when sites are involved that capture a larger amount of drug while changes on low deposition affect the MmCSRS less. This behavior, suggested from Fig. 3, is more quantitatively expressed in Fig. 4, which depicts a linear relationship between the MmCSRS and the NSDRS (Eq. 3, a measure for differences in mean deposition between two CI profiles after normalization). This behavior is expected from Eq. 1 and was similarly observed for the originally proposed CSRS (5). It is generally believed that such a behavior is desirable for APSD equivalence testing as the smaller effect of low deposition sites (often related to non-product factors, such as challenges of the analytical procedure to quantify potential differences in the ng range) on the overall MmCSRS are expected to be much less relevant for BE of the T and R OIDPs. Contrary to univariate approaches such as the mCSRS, procedures employing a site by site analysis of cascade impactor data using standard BE test methodology for a given stage (*e.g.*, proposed by EMA (9,10)) do not possess this characteristic as they will even fail different batches of the same R OIDP, in part, due to the high variability (in terms of CV%) associated with the low deposition sites. Furthermore, such methods face statistical challenges because of necessity of perform multiple comparisons. Overall, an equivalence test based upon the MmCSRS might have the potential to be more robust, as above factors have a smaller effect on MmCSRS (differences of low deposition stages) or are not applicable (challenges because of multiple comparisons).

To further evaluate the behavior of the MmCSRS, differences in the mean deposition between the T and R CI profiles were still limited to one site. However, T and R CI profiles were also allowed to differ in their variability. Therefore, the variability was modulated on all deposition sites, which was believed as being more realistic than changing the variability of a single site only. As expected from the simulations where T and R CI profiles had the same variability (Fig. 3), the MmCSRS increased with increasing difference in mean deposition (compare Fig. 5 for 30% difference with those observed for a 10% difference in the

Fig. 6. Behavior of the MmCSRS (displayed as average of the 20,000 samples (y-axis)) when T and R CI profiles differ from each other on multiple sites in their mean deposition and variability for CI profile M2 (Fig. 2) without inter-site correlation; x-axis, variability of the R CI profiles (displayed as the squared inverse of the coefficient of variation (CV%)), T and R CI profiles differed from each other by $\pm 5\%$, 10% , 15% , 20% , 25% , or 30% on all deposition sites such that the cumulative total mass of all deposition sites was identical; *circles*, T and R CI profiles had the same variability on all deposition sites; *triangles*, the variability on the sites of T CI profiles was half of that of the R CI profiles; *plus sign*, the variability on the sites of T CI profiles was twice as large as that of the R CI profiles

supplemental material Fig. S2 for the same deposition site). More importantly, MmCSRS was also sensitive to changes in the variability of the T product, as a higher T variability (*i.e.*, an increased T/R ratio) resulted in an increase in MmCSRS (Fig. 5). This increase was almost linear and seen for all deposition sites under consideration (site 1, 3, 7, and 11; see Fig. 1 for shape of CI profile) (Fig. 5). These observed behaviors were expected from the computational form of the mCSRS (Eq. 1), as the value of the cumulative numerator expression is driven by the overall difference in mean T and R deposition (the larger this difference, the larger the expression,

$$\sum_{i=1}^p \frac{(T_{ij} - \bar{R}_i)^2}{\bar{R}_i}$$

the larger the MmCSRS; Fig. 3). This expression is also affected by the degree of T variability (the larger T variability, the larger the numerator expression,

$$\sum_{i=1}^p \frac{(T_{ij} - \bar{R}_i)^2}{\bar{R}_i}$$

the larger the MmCSRS). For both cases, the denominator is not affected. Even though only differences

between T and R CI profiles of 10% and 30% were evaluated, it seems reasonable to extrapolate these results to any difference between T and R CI profiles that is relevant in the context of equivalence testing. Overall, the fact that MmCSRS increases with increasing T/R variability ratio is desired as it penalizes and rewards the T product for having an increased and decreased variability, respectively.

Figure 5 also indicates that MmCSRS depends on the variability of the R product (not only on the T/R ratio), as MmCSRS for a constant T/R ratio differs depending on whether the R product shows “high,” “normal,” or “low” variability (Fig. 5). Whereas in previous simulations (differences in mean deposition and differences in T variability), MmCSRS behavior was driven by differences in the numerator, Eq. 1 readily suggests that the sensitivity of MmCSRS to differences in R variability is driven solely by the denominator expression,

$$\sum_{i=1}^p \frac{(R_{ik} - \bar{R}_i)^2}{\bar{R}_i}$$

This cumulative denominator expression is only capturing variability of in the R product (per definition there cannot be a difference in mean deposition between R products). The cumulative denominator term will increase with increasing R

Fig. 7. Behavior of the MmCSRS (displayed as average of the 20,000 samples (y-axis)) when T and R CI profiles differ from each other on multiple sites in their mean deposition and variability for CI profile M5 (Fig. 2) with inter-site correlation; x-axis, variability of the R CI profiles (displayed as the squared inverse of the coefficient of variation (CV%)), T and R CI profiles differed from each other by $\pm 5\%$, 10% , 15% , 20% , 25% , or 30% on all deposition sites such that the cumulative total mass of all deposition sites was identical; *circles*, T and R CI profiles had the same variability on all deposition sites; *triangles*, the variability on the sites of T CI profiles was half of that of the R CI profiles; *plus sign*, the variability on the sites of T CI profiles was twice as large as that of the R CI profiles

variability and as consequence the MmCSRS will decrease. Reduced R variability will result in an increase in MmCSRS.

Hence, the observed increase in MmCSRS with decreasing variability of the R product while the difference between T and R CI profiles and the T/R variability ratio were kept constant is a consequence of the computational form of the mCSRS. The implications of this behavior for equivalence testing of CI profiles are discussed below.

Multiple Site Mean Differences

The analysis of the effect of differences between T and R CI profiles on multiple sites on the MmCSRS was restricted to eight sites because of the following reasons. The eight sites could possibly represent the sites that are comprised in the definition of the ISM of an Andersen CI (4). A CI profile comparison test for establishing equivalence between T and R ODPs would most likely be applied to those sites only since differences on non-ISM sites, which the MmCSRS would be sensitive to, are not very relevant for lung deposition, and could be detected by other means, for example, by testing the equivalence of the single actuation content and ISM before a MmCSRS based profile comparison test is applied. In addition, we showed in the previous publication, that the MmCSRS behavior was not dependent on the number of stages included in the analysis of identical T and R profiles (2).

We assumed within these simulations that the deposited mass on the eight deposition sites (ISM sites) was constant, a likely pre-condition asked for within future APSD tests. It should be emphasized that only such conditions, insured through preliminary statistical tests, would allow truly testing for differences between two CI profiles in mean deposition and/or variance of sites that are considered more relevant for lung deposition.

The effects of multiple site differences on the behavior of the MmCSRS under these conditions were assessed for a variety of CI profiles (M1–10; Fig. 1) by changing mean deposition and variability without changing total cumulative deposition (*i.e.*, T and R CI profiles had an identical total deposition on all eight sites; see above), as shown for two scenarios in Table I.

As expected, the MmCSRS increased with increasing differences in mean deposition (Figs. 6 and 7, comparison of MmCSRS for a constant CV% across panels). This confirmed the results obtained for single site mean differences between two CI profiles (*see above*) but without the influence of differences in cumulative deposition. Similarly, it could be seen from these simulations that for a given difference in mean deposition the MmCSRS increases again with increasing variability of the T product (*see above*). These relationships were not surprising, but validated the expected behavior of the MmCSRS.

In these simulations, we included situations that incorporated an inter-site correlation (the characteristic of one site affecting the behavior of another site), as this behavior cannot

Table II. Results of the Analysis of the Effect of Multiple Site Changes on the MmCSRS for the CI Profiles M1–10 (Fig. 2) for Scenarios with and Without Inter-site Correaltion, for the 1,080 Scenarios for which T and R CI Profiles Had the Same Variability

P	Diff	No inter-stage correlation					Inter-stage correlation				
		R^2	β_0	β_1	NSD	β_1/NSD	R^2	β_0	β_1	NSD	β_1/NSD
M1	5	0.9998	1.08	30.3	3.1	9.7	0.9999	1.09	42.5	3.125	13.6
M1	10	1	1.08	126.1	12.5	10.09	1	1.1	170.3	12.5	13.6
M1	15	0.9999	1.08	289.7	28.1	10.3	1	1.12	380.4	28.125	13.5
M1	20	1	1.09	519.5	50.0	10.39	1	1.14	673.3	50	13.5
M1	25	1	1.09	815.0	78.1	10.43	1	1.17	1,046.1	78.125	13.4
M1	30	1	1.1	1,177.1	112.5	10.46	1	1.22	1,503.7	112.5	13.4
M2	5	0.9998	1.08	30.6	3.3	9.42	0.9999	1.09	42.4	3.25	13.1
M2	10	0.9999	1.08	127.7	13.0	9.82	1	1.1	169.5	13	13.0
M2	15	0.9999	1.08	291.8	29.3	9.98	1	1.11	378.4	29.25	12.9
M2	20	0.9999	1.08	524.4	52.0	10.08	0.9999	1.14	667.1	52	12.8
M2	25	1	1.09	824.8	81.3	10.15	1	1.17	1,044	81.25	12.9
M2	30	1	1.1	1,190.0	117.0	10.17	1	1.21	1,496.9	117	12.8
M3	5	0.9995	1.09	32.2	4.3	7.58	0.9998	1.09	46	4.25	10.8
M3	10	0.9997	1.09	135.6	17.0	7.98	1	1.1	183.5	17	10.8
M3	15	0.9998	1.08	315.3	38.3	8.24	1	1.11	411.5	38.25	10.8
M3	20	0.9999	1.07	569.3	68.0	8.37	1	1.13	731.2	68	10.8
M3	25	0.9999	1.07	899.2	106.3	8.46	1	1.16	1,141.7	106.25	10.8
M3	30	0.9999	1.07	1,304.5	153.0	8.53	1	1.2	1,642.5	153	10.7
M4	5	0.9991	1.1	37.8	8.5	4.43	0.9996	1.11	40.6	8.54	4.8
M4	10	0.9983	1.09	175.9	34.2	5.15	0.9993	1.09	182.1	34.16	5.3
M4	15	0.9984	1.04	436.2	76.9	5.68	0.9991	1.04	446.8	76.86	5.8
M4	20	0.9987	0.97	818.5	136.6	5.99	0.9994	0.99	827.3	136.64	6.1
M4	25	0.9992	0.91	1317.6	213.5	6.17	0.9996	0.95	1,328.2	213.5	6.2
M4	30	0.9995	0.85	1,935.3	307.4	6.29	0.9998	0.9	1,944.9	307.44	6.3
M5	5	0.9988	1.09	32.4	4.6	7.1	0.9999	1.09	42.2	4.5625	9.3
M5	10	0.9996	1.08	138.5	18.3	7.59	1	1.09	171.6	18.25	9.4
M5	15	0.9998	1.08	321.5	41.1	7.83	1	1.1	387.8	41.0625	9.4
M5	20	0.9999	1.07	582.5	73.0	7.98	1	1.11	692.5	73	9.5
M5	25	0.9999	1.06	918.8	114.1	8.06	1	1.14	1,081.7	114.0625	9.5
M5	30	0.9999	1.06	1,331.2	164.3	8.1	1	1.17	1,558.2	164.25	9.5
M6	5	0.9996	1.1	36.0	5.8	6.24	0.9999	1.1	58.4	5.77	10.1
M6	10	0.9996	1.09	154.7	23.1	6.7	1	1.11	236.3	23.08	10.2
M6	15	0.9996	1.07	361.9	51.9	6.97	1	1.11	537	51.93	10.3
M6	20	0.9998	1.05	661.4	92.3	7.16	1	1.12	957.7	92.32	10.4
M6	25	0.9999	1.02	1,046.2	144.3	7.25	1	1.13	1,496	144.25	10.4
M6	30	0.9999	1	1,516.7	207.7	7.3	1	1.16	2,156.8	207.72	10.4
M7	5	0.9989	1.09	33.1	5.3	6.3	0.9997	1.1	39.8	5.25	7.6
M7	10	0.9995	1.09	141.9	21.0	6.76	0.9999	1.09	166.1	21	7.9
M7	15	0.9996	1.07	334.5	47.3	7.08	1	1.09	383.4	47.25	8.1
M7	20	0.9998	1.06	607.2	84.0	7.23	1	1.08	693.8	84	8.3
M7	25	0.9998	1.05	963.5	131.3	7.34	1	1.09	1,089.7	131.25	8.3
M7	30	0.9999	1.04	1,398.4	189.0	7.4	1	1.1	1,579.5	189	8.4
M8	5	0.9994	1.1	35.1	6.7	5.23	0.9995	1.1	42.3	6.71	6.3
M8	10	0.9993	1.09	157.0	26.8	5.85	0.9998	1.09	181	26.84	6.7
M8	15	0.9994	1.06	374.2	60.4	6.2	0.9998	1.08	425.1	60.39	7.0
M8	20	0.9995	1.03	691.2	107.4	6.44	0.9998	1.06	775.1	107.36	7.2
M8	25	0.9997	1	1,098.9	167.8	6.55	0.9999	1.05	1,224.2	167.75	7.3
M8	30	0.9998	0.98	1,603.8	241.6	6.64	1	1.05	1,775.7	241.56	7.4
M9	5	0.9988	1.1	37.8	8.2	4.62	0.9997	1.11	40.8	8.19	5.0
M9	10	0.9987	1.09	171.6	32.8	5.24	0.9994	1.09	183.4	32.76	5.6
M9	15	0.9985	1.04	421.6	73.7	5.72	0.9993	1.05	442.8	73.71	6.0
M9	20	0.9991	0.99	786.0	131.0	6	0.9994	1.01	824	131.04	6.3
M9	25	0.9994	0.93	1,267.1	204.8	6.19	0.9996	0.97	1,314.8	204.75	6.4
M9	30	0.9996	0.88	1,857.6	294.8	6.3	0.9998	0.93	1,928.2	294.84	6.5
M10	5	0.9997	1.09	31.4	3.8	8.37	0.9999	1.09	42	3.75	11.2
M10	10	0.9999	1.09	131.1	15.0	8.74	1	1.1	166.4	15	11.1
M10	15	0.9999	1.08	302.3	33.8	8.96	1	1.11	374.3	33.75	11.1
M10	20	0.9999	1.08	545.4	60.0	9.09	1	1.13	664	60	11.1
M10	25	1	1.08	860.1	93.8	9.17	1	1.16	1,035.6	93.75	11.1
M10	30	1	1.08	1,243.7	135.0	9.21	1	1.19	1,486.3	135	11.0

The averages of the MmCSRS were regressed against the squared inverse of the coefficient of variation of the R product separately for each of the groups (defined by the CI profile M1–10 and the difference in mean deposition between T and R CI profiles) P profile, $Diff$ percentage difference between test and reference CI profile on all stages, R^2 coefficient of determination, β_0 estimated intercept parameter, β_1 estimated slope parameter, NSD normalized squared difference between test and reference CI profiles

Fig. 8. Analysis of effect of differences between T and R CI profiles in mean deposition and/or variability on multiple sites for the scenarios where T and R CI profile had the same variability; *top-left panel*, estimated intercepts from simple linear regression of MmCSRS against SqInCV on all deposition sites (y-axis) plotted against the NSD (Eq. 4) between T and R CI profiles (x-axis); *top-right panel*, estimated slopes from simple linear regression of MmCSRS against SqInCV on all deposition sites (y-axis) plotted against the NSD between T and R CI profiles (x-axis); *bottom-left panel*, estimated intercepts from simple linear regression of MmCSRS against SqInCV on all deposition sites for scenarios without inter-site correlation (y-axis) plotted against estimated intercepts from simple linear regression of MmCSRS against SqInCV on all deposition sites for scenarios with inter-site correlation (x-axis); *bottom-right panel*, estimated slopes from simple linear regression of MmCSRS against SqInCV on all deposition sites for scenarios without inter-site correlation (y-axis) plotted against estimated slopes from simple linear regression of MmCSRS against SqInCV on all deposition sites for scenarios with inter-site correlation (x-axis); r , correlation coefficient; β_0 , intercept; β_1 , slope; RSQ , coefficient of determination

be excluded from “real” cascade impactor studies, and the behavior of the MmCSRS under those conditions warranted further evaluations. For any of the CI profiles M1–10 and regardless of the inter-site correlation structure, the MmCSRS increased as the variability (in terms of CV) of the R CI profiles decreased even for the situation where the T/R variability ratio was kept constant (Figs. 6 and 7). This behavior of the MmCSRS for multiple site differences is similar to that for single site changes where the MmCSRS was increased for less variable R products in spite of a constant T/R variability ratio and was explained above. However, this dependency of the MmCSRS on the variability of the R product for a constant T/R variability ratio and a constant difference in mean deposition between T and R CI profiles, even for the situation where the cumulative deposited drug amount was constant, have the following significant implication. A rigid cut-off value for an APSD equivalence test based upon a fixed MmCSRS is not feasible, and needs to be

scaled by the variability of the R CI profile, in order to be able to consistently discriminate equivalent and inequivalent CI profiles (*i.e.*, a certain difference (*e.g.*, 20%) between T and R CI profiles on all of the deposition sites under consideration yields a different MmCSRS dependent on the variability of the R product).

The relationship between MmCSRS and variability of the R product could be identified as straight line when plotting the MmCSRS against the SqInCV when T and R CI profiles had the same variability or the T product was less variable (Figs. 6 and 7). However, this linear relationship could not be observed when the T product was more variable and the variability of both products became high (Figs. 6 and 7).

Since for construction of a APSD equivalence test the understanding of the behavior of the MmCSRS where T and R CI profiles have the same variability is considered as most important, simple linear regression of the average of the 20,000 MmCSRSs against SqInCV separately for each CI

profile M1–10 and each difference between T and R CI profile in their mean depositions was performed only for those scenarios and yielded a perfect linear relationship ($R^2 > 0.999$) (Table II).

The estimated intercept parameters seemed to be independent of the inter-stage correlation structure and were close to one regardless of the NSD (Eq. 4, a measure for the difference between T and R CI profiles in mean deposition) and the shape of the CI profiles (*i.e.*, M1–10) (Table II; Fig. 8, top-left and bottom-left panels). The estimated slope parameters increased as the NSD increased and were highly correlated ($R^2 = 0.89$) (Fig. 8, top-right panel). Therefore, the estimated slope parameters represent a good measure for quantification of the differences between T and R CI profiles in their mean site depositions.

Moreover, after scaling the slope parameters on their respective NSD, the scaled metrics (slope/NSD) were similar within a certain CI profile (Table II). On the other hand, there were still some differences in the scaled metrics between the ten CI profiles M1–10 (Table II). These differences in the scaled metrics are a consequence of the fact that a certain difference in mean deposition does not result in the same NSD for different profiles (*e.g.*, a 30% difference in mean deposition yielded an NSD of 112.5 and 153 for the CI profiles M1 and M10, respectively; Table II) and result in a larger weighting of sites with an increased depositions. The positive implications of this characteristic for APSD equivalence testing were discussed above.

In spite of being highly correlated ($R^2 = 0.97$), the estimated slope parameters were smaller for the cases without any inter-site correlation compared with those with inter-site correlation (Table II; Fig. 8, bottom-left panel). Specifically, for a specific SqInCV of the R CI profile, the MmCSRS was decreased in average by $-8.76 + 0.86 \cdot \text{SqInCV}$ for the zero inter-site correlation case compared to that with inter-site correlation. The implications and practical relevance of this for APSD equivalence testing need to be further evaluated by simulation studies.

In summary, for multiple site differences, the observed differences in MmCSRS were dependent on the difference in mean deposition between T and R CI profiles, the T/R variability ratio, the variability of the R product, and the inter-stage correlation structure. For the 1,080 scenarios where T and R CI profiles had the same variability, the differences in MmCSRS is a function of the NSD (as a measure for difference in mean deposition) and the SqInCV (as a measure of the variability of the R product) of the R CI profile. Since the value of the MmCSRS is perfectly correlated with the SqInCV of the R CI profile for any of the ten CI profiles M1–10, the SqInCV of the R CI profiles appears to be a good choice as a metric for reference variance scaling. A more detailed analysis of the reference variance scaling approach will be given in a forthcoming article (part III).

It should be noted that all CI profiles were constructed such that the CV was the same on all deposition sites. However, the assumption of identical CVs on all deposition sites is not expected for real CI profiles. In fact, the variability (expressed as CV) is expected to be increased on low deposition sites compared with that for high deposition sites. However, setting the CVs to be equal on all eight deposition sites was necessary for being able to systematically evaluate and understand the behavior of the MmCSRS when T and R CI profiles differ from

each other on multiple deposition sites while maintaining a manageable level of complexity. The behavior of MmCSRS in situations where the CI profiles have an increased variability on low deposition sites warrants further analysis. Furthermore, simulation analysis is required to evaluate which metric is the best measure for the variability when the CI profiles have different CVs on the deposition sites. These will be addressed in the forthcoming article (part III).

All results are based upon the assumption that a sample of 30 T and R CI profiles is obtained, which is in accordance with the June 1999 *Draft Guidance for Industry: Bioavailability and Bioequivalence Studies for Nasal Aerosols and Nasal Sprays for Local Action* (3). It should be noted that the behavior of the MmCSRS has not been studied when a different sample size is obtained. However, since the expected “average” behavior of the MmCSRS is studied in this article, it is not expected that a different sample size would affect the conclusions here. The sample size will, of course, influence the width of confidence intervals for the MmCSRS and will be discussed in a forthcoming article (part III).

It should also be remarked that the application of the MmCSRS is not limited to the comparison of CI profiles and may potentially be applied to other multivariate equivalence testing problems.

CONCLUSIONS

For (a) constant difference(s) between T and R CI profiles on a single or multiple deposition sites, the MmCSRS increases as the magnitude of deposition on the site(s) on which the difference(s) occur(s) becomes larger. Thus, the MmCSRS gives a larger weight to differences on sites with increased deposition. This characteristic was considered as beneficial for APSD equivalence/profile comparison testing, as it should decrease the likelihood of failing identical products due to increased variability on low deposition sites (*e.g.*, constructing univariate confidence intervals on each deposition site) (9,10). Most importantly, it was demonstrated that a cut-off (critical) value for APSD equivalence testing based on the MmCSRS needs to be scaled on variability of the R product for consistently being able to discriminate equivalent from inequivalent CI profiles.

REFERENCES

1. Lee SL, Adams WP, Li BV, Conner DP, Chowdhury BA, Yu LX. *In vitro* considerations to support bioequivalence of locally acting drugs in dry powder inhalers for lung diseases. *AAPS J.* 2009;11:414–23.
2. Weber B, Hochhaus G, Adams W, Lionberger R, Li B, Tsong Y, *et al.* A stability analysis of a modified version of the chi-square ratio statistic: implications for equivalence testing of aerodynamic particle size distribution. *AAPS J.* 2013;15:1–9.
3. Food and Drug Administration Center for Drug Evaluation and Research (CDER). Guidance for industry - bioavailability and bioequivalence studies for nasal aerosols and nasal sprays for local action (Draft Guidance), June 1999. <http://www.fda.gov/ohrms/dockets/ac/00/backgrd/36099b1.pdf>. Accessed 13 June 2012
4. Christopher D, Adams W, Amann A, Bertha C, Byron PR, Doub W, *et al.* Product quality research institute evaluation of cascade impactor profiles of pharmaceutical aerosols, part 3: final report on a statistical procedure for determining equivalence. *AAPS PharmSciTech.* 2007;8:65–74.

5. Christopher D, Adams WP, Lee DS, Morgan B, Pan Z, Singh GJ, *et al.* Product quality research institute evaluation of cascade impactor profiles of pharmaceutical aerosols: part 2—evaluation of a method for determining equivalence. *AAPS PharmSciTech.* 2007;8:E39–48.
6. Izenman AJ. *Modern multivariate statistical techniques—regression, classification, and manifold learning.* 1st ed. New York: Springer; 2008. p. 56–61.
7. Christensen R. *Plane answers to complex questions.* 4th ed. New York: Springer; 2011. p. 5–8.
8. Genz A, Bretz F, Miwa T, Mi X, Leisch F, Scheipl F, *et al.* Multivariate normal and t distributions. <http://cran.r-project.org/web/packages/mvtnorm/mvtnorm.pdf>. Accessed 07/02/2012.
9. Evans C, Cipolla D, Chesworth T, Agurell E, Ahrens R, Conner D, *et al.* Equivalence considerations for orally inhaled products for local action-ISAM/IPAC-RS European workshop report. *J Aerosol Med Pulm Drug Deliv.* 2012;25:117–39.
10. European Medicines Agency. Guideline on the requirements for clinical documentation for Orally Inhaled Products (OIP) including the requirements for demonstration of therapeutic equivalence between two inhaled products for use in the treatment of Asthma and Chronic Obstructive Pulmonary Disease (COPD) in adults and for use in the treatment of Asthma in children and adolescents. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003504.pdf. Accessed 13 June 2012.