Evaluation Method of Insoluble Particulate Matter in Biotechnological Products (Biopharmaceuticals) Drug Substances/Drug Products by Flow Imaging Method 〈*G3-17-182*〉

（フローイメージング法によるバイオテクノロジー応用医薬品(バイオ医薬品)原薬／製剤中の不溶性微粒子の評価法〈*G3-17-182*〉）

Biotechnological products (hereinafter referred to as "biopharmaceuticals") may contain, insoluble particulate matter such as protein aggregates generated by proteins aggregating themselves, in addition to exogeneous materials, manufacturing-process-derived materials and extractable substances from the formulation composition or the primary container. Evaluation and control of particulate matter contained in injections play an important role in assuring the quality of final products. For the protein aggregates, more rigorous evaluation and control is required because of immunogenicity concern over protein drug products.

The flow imaging method is a technique to count particulates contained in a solution and measure their size distribution, and evaluate their morphological and optical properties, by analyzing the numerical information converted from the digital images which are captured continuously on the sample solution flowing into a flow cell. The light obscuration particle count test may not detect protein aggregates at all or underestimate their particle size due to the difference in refractive index from water being so small. This is because the particle size is calculated by a particle size response curve based on polystyrene standard particles with a high refractive index. The flow imaging method has been, on the other hand, shown to be less sensitive to refractive index difference between the particles and the dispersion solvent than the light obscuration particle count test. Furthermore, by evaluating the morphological and optical properties it is also possible in some cases to classify protein-aggregates, silicon oil, air bubbles and other insoluble particulates. Quantitative evaluation of the number of particles and characterization of the particles by the flow imaging method is a useful evaluation method for insoluble particulates in therapeutic protein injections. In this general information, evaluation methods for insoluble particulates contained in biopharmaceuticals including therapeutic protein injections are mainly described.

1. Principles of Measurement

The apparatus generally consists of a sample port, a flow cell which is the area for capturing images, flow path tubes for connection, a pomp (a tube pomp or a syringe pomp), an optical system including a light source, a camera as an imaging instrument, and an image analyzer for the captured images. A sample solution flowing into the flow-cell is irradiated by light from the source and is captured by the imaging instrument. A measurable particle size depends on the thickness of the flow-cell, the magnification of the objective lens and the performance of the camera, and in most cases the measurement range is approximately 2 to 100 μm. The particle image data is processed by the image analyzer and evaluated for the shape and optical properties of each particle by recognizing the boundary of each particle in the image based on, for example, the contrast of the particles against the image background. The particle concentration is obtained by dividing the particulate count by the measured volume.

2. Measurement

2.1. Instrument

General procedure for the measurement is as follows. Employ the magnification of the objective lens according to the size of the particles to be measured, which is usually 4 to 20-fold. Clean the flow-cell in advance and ensure that there are no particle remaining in the flow-cell. For cleaning the flow-cell use particulate-free water or use, as necessary, detergent, diluted sodium hydroxide aqueous solution and ethanol, etc. Thereafter, focus the instrument appropriately following its operation procedure. Set required measurement parameters (flow rate, sample volume, image acquisition frequency, particle identification threshold against the background, etc.) for each instrument. An image acquisition efficiency is defined as the rate of the portion of the solution for which the images are analyzed to the total introduced into the flow-cell. For instruments that allow the setting of image acquisition efficiency, the efficiency is calculated from the sample volume, flow rate, and image acquisition frequency (image acquisition efficiency＝image acquisition frequency (frames/s) × measured volume per image (mL/frame)／flow rate (mL/s) × 100 (%). The settings should be properly performed so that same particles are not counted in multiple times and that the sample volume for actual measurement is adequate. Where the area to be measured can be set, accuracy in counting particles can be verified by measuring a particle count reference standard. Due to the nature of the instrument, a particle image with missing part may be captured due to part of particles being out of the measurement area. Handling of partially captured images of particles should be stipulated in advance.

2.2. Operating method

The measurement should be carried out under conditions limiting particulate contamination, preferably, in a clean cabinet with laminar flows, etc. Gently shake the sample thoroughly, swirling the container slowly for example, so that the particles in the sample are uniformly dispersed. When opening the container, clean the outer surface of the container opening with *particle-free water* and remove the closure cautiously to avoid contamination of the contents. When

measuring particulates in solution, caution is required not to generate bubbles or new aggregates during the operation. If necessary, allow the container to stand under ambient pressure or reduced pressure for the moment to eliminate air bubbles. Sonication is not appropriate as it may cause aggregation or denaturation of proteins. The volume of sample to be introduced into the instrument is determined considering the sample volume and the tare volume. The sample volume is determined in an adequate volume considering the properties of the sample, the image acquisition efficiency, and the precision required for the analysis. If necessary, such as when the sample has a high viscosity or a large number of particles, it would be possible to dilute the sample subject by confirming a dilutional linearity. The number of the measurements should be determined appropriately based on the performance of the instrument and the properties of the sample.

When using an instrument that can set the threshold value individually, confirm in advance that the particle borders are properly recognized, as the threshold value impacts the results of analysis significantly. It is also advisable to verify that the particle shapes are correctly evaluated, and that noise is not misconstrued as a particle by using an actual sample, a degraded actual sample, or particle standards prepared to imitate protein aggregates. When comparing the data acquired at different threshold values, the impact of the difference in threshold values on the results of measurements should be duly considered.

3. Image analysis

The sizes of detected particles are often represented by an equivalent circle diameter (the diameter of a circle having an area equivalent to the projected area of the particle). Other than the circular equivalent diameter, a sphere equivalent diameter or a Feret's diameter can also be used. Comparison of the particle sizes represented by such different particle definitions needs some caution.

While the counting of particulates by the flow imaging method is the main object in this general information, the particle image may provide an estimate as to the origin, or the particles may be classified according to the features of the image. The main parameters to feature the particle properties, obtained as a result of the image analysis, include morphological parameters such as, in addition to particle size, area, particle perimeter, aspect ratio, circularity, etc., as well as optical parameters such as brightness, standard deviation of brightness within the particles. Using these parameters, it is possible to classify the particles in the sample by the origin, such as, for example, silicon oil droplets derived from the container. Aspect ratio, roundness, perimeter, length, average and standard deviation of brightness are used to classify silicon oil droplets. Combine some of these parameters, set an optimal threshold of each parameter, and sieve step by step. A classification model can be established using sufficient image data accumulated, and used to classify the detected particles by their origin, by applying to image data acquired by the same instrument. As these parameters, however, depend on the definition formula embedded in the imaging instrument and analysis software, as well as on the image analyzer system and the measurement conditions, the measured values may differ depending on the resolution, pixel number, and focusing method. Further to identify the origin, it should be necessary to use other appropriate technology such as micro-Raman spectroscopy which provides information on molecular structure and composition.

4. Validation of analytical method

Validation of an analytical method is to demonstrate the validity of the method by demonstrating conformity to the pre-defined criteria for validation characteristics, such as accuracy, precision, specificity (selectivity) in general. The validation characteristics to be evaluated depend on the purpose of the test which uses the analytical method concerned. When a test method is to perform counting of insoluble particulates in pharmaceuticals, it would be difficult to conduct method validation in a similar manner as for usual quantitative assays, because there is no control sample with known accuracy that reflects an actual sample, making accuracy evaluation difficult, and the particulates contained in the actual samples of a drug product or a drug substance are distributed widely and heterogeneously in respect of particle sizes. The following validation characteristics are evaluated to demonstrate the validity of the method, using, for example, polystyrene particle standard with certified average particle size or polystyrene particle count reference standard with certified average particle size and particle concentration. The particle sizes and concentrations of the particle standard and particle count reference standard to be used should be appropriately determined considering the particle concentration and particle size distribution in the actual samples and specification values. Multiple particle standards having different particle sizes may also benefit the evaluation of analytical method performance. The particle size distribution or the number of particles of particle standards to be used should be certified and quality-assured by an appropriate organization. In addition, silica particles or polymethylmethacrylate particles, both having a low refractive index, may serve as suitable model particles for protein-aggregates. These particles may therefore be useful in confirming if the particle size to be measured varies due to a small difference in refractive index between the model particle and the solvent, by utilizing the sample prepared by adding them to the solution with the same formulation composition as that of the actual sample solution to be tested.

Example of validation procedure for counting the number of particles by flow imaging method.

Accuracy: Measure 5, 10, and 25 μm polystyrene particle count reference standards and verify that the results obtained are within the certified particle size and particle concentration ranges.

Precision: Evaluate repeatability and intermediate precision. Add 5, 10 or 25 μm standard particles to particle-free water or a solution consisting of the same formulation composition as that of the sample to be tested, to prepare samples for 3 levels of the particle concentrations for each standard particle. Measure each sample 3 times for repeatability. Using similarly prepared samples, measure the samples at least on different days and by different operators under the same conditions to calculate intermediate precision.

Linearity: Add 5, 10 or 25 μm standard particles to particle-free water or a solution consisting of the same formulation composition as that of the sample to be tested, and evaluate the linearity, for example, at 5 levels of particle concentration.

Specificity: When the particles are to be classified using classification model or as otherwise required, verify that the classification is properly performed, using a degraded sample or an actual sample with a target analyte added.

5. Assuring instrument performance

5.1. Calibration

The particle sizes and the number of particles calculated by the flow imaging method are absolute values based on the principle of the measurement instead of relative values calculated from the measured values of particle standards. Therefore, confirm using particle count reference standards that the instrument is operating correctly, and adjust the settings if necessary. It is essential to confirm that the optical system operates appropriately in respect of focusing, brightness of the light source, etc. In addition, since the performance of the pump can also affect the measurement results, the flow rate should be adjusted and checked. For calibration of the instruments, use a polystyrene particle count reference standard and a polystyrene particle standard, with the particle size distribution and number of particles assured based on the absolute methods and certified by an appropriate organization.

5.2. System Suitability

To confirm in advance of the measurement that the instrument is in appropriate operation condition and has been adequately cleaned, it is recommended to set the following system suitability.

Confirm that the measured values (particle size and number of particles) obtained for an appropriate particle standard are within the pre-defined range. Confirm that the number of particles in filtered water (prepared immediately before use) is not more than the specified value. Set an appropriate particle size range according to the purpose. When the number of particles in the filtered water falls outside the appropriate range, repeat preparation of water to be used and cleaning of the instrument, and re-measure.