Application of Micro-Flow Imaging (MFI™) to The Analysis of Particles in Parenteral Fluids

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October 2006
Ottawa, Canada
Summary

The introduction of a growing number of targeted protein-based drug formulations poses a significant challenge to the instrumentation technologies traditionally used for sub-visible and visible particle population analysis in parenteral fluids. US Pharmacopeia Chapter 788 (USP788) specifies maximum concentrations of sub-visible particles having equivalent a circular diameter greater than or equal to 10µm and 25µm as determined by light obscuration or, when applicable, by microscopic analysis following filtration. Since proteinaceous particles are highly transparent when compared to the polystyrene (PS) beads used for obscuration instrument calibration, they may be grossly undersized or missed entirely using this technique. The result is that a formulation may be measured as meeting USP788 requirements when, in fact, it does not.

The limitations of light obscuration instruments have led to an interest in alternative technologies which are less dependent on particle properties and can provide additional information on particle parameters.

This paper will describe the application of Micro-Flow Imaging (MFI™) technology to the analysis of parenteral particle populations in both the sub-visible and visible range. The instrument’s performance with respect to its range of measurement parameters which complement this application will be outlined. A direct comparison will show that MFI can detect and measure particle concentrations in proteinaceous samples which are one or more orders of magnitude higher than the standard method, and that this effect is most pronounced for the larger particles of interest in USP788.

The capability of the instrument to provide and analyze images of each particle will be shown. The value of the additional information provided by particle morphology data in assessing clinical outcome and in assisting to identify particle origin for further process control, quality control, diagnostics and troubleshooting will be discussed.

1.0 Introduction

USP788 (Particulate Matter in Injections) describes physical tests to be performed for the purpose of enumerating sub-visible particles within specific size ranges contained within parenteral fluids. USP788 identifies light obscuration and microscopy as the methods for determining particle counts within the specified size ranges.

Light obscuration is an indirect measurement technique. When a particle transits the measurement zone an optical beam is obscured with a resulting change in signal strength at the detector. This signal change is then equated to a particle’s equivalent circular diameter (ECD) based on a calibration curve created using polystyrene (PS) spheres of a known size. To the extent that particles in intravenous solutions are composed of different materials and are often far from spherical, errors in sizing and counting are inevitable. Particles which are composed of highly transparent materials can be grossly undersized and, as a result, the concentration of larger particles is underestimated.

A 2003 Workshop on Particle Size Analysis was co-sponsored by the American Association of Pharmaceutical Scientists (AAPS), the Food and Drug Administration (FDA) and the United States Pharmacopeia (USP). The subsequent report1 states:

“The measurement and expression of particle size is intimately bound with the shape and morphology of the constituent units that make up the ensemble of particles. The difficulties encountered when relating empirical information derived using different methods would not exist if the component particles were spherical. In the real world of pharmaceutics, particles are rarely (if ever) spherical, and consequently it is important to understand the importance of particle shape and morphology.”

1 AAPS PharmaSci 2004; 6 (3) Article 20, Page 3
This paper explores the application of MFI to the analysis of subvisible and visible particles in parenteral fluids. The following measurement parameters are considered:

- Section 3.1  Sensitivity for highly transparent particles
- Section 3.2  Dependence on particle material type
- Section 3.3  Sizing accuracy and repeatability
- Section 3.4  Concentration accuracy and repeatability
- Section 3.5  Visible/dense particle introduction
- Section 3.6  Very low concentrations of larger particles
- Section 3.7  Shape Analysis and Particle Morphology

2.0  Micro-Flow Imaging (MFI)

MFI is a flow microscopic technology which operates by capturing images of suspended particles in a flowing stream. Different magnification set-points are available to suit the desired particle size range and image quality. The images are used to produce a particle database including count, size, transparency and shape parameters. The database can be interrogated to produce particle size distributions and isolate sub-populations using any measured parameter. Particle images are available for verification, further investigation and analysis. The sample volume in each frame is precisely defined by the flow cell geometry so particle concentrations are determined absolutely. No calibration by the user is required and all critical system parameters are automatically pre-set to eliminate run-to-run and user dependent variations.

For parenteral fluids, the primary interest is in detecting and measuring relatively large particles (>2µm). Therefore the low magnification (x5) option was selected. Very large (>70µm) particles can pose particular problems for sample introduction by aspiration due to particle densities and settling velocities. MFI includes a “top-down” gravity-assisted sample introduction method to address this problem. The sample introduction module permits the MFI instrument to analyze large particles which cannot be reliably suspended and aspirated. This sample introduction methodology is equally effective for small and large particles. The equipment setup is shown in Figure 1.

![Figure 1 - Isometric View of Sampling System](image-url)
A volume sampling speed of 1ml per 5 minutes is typically used. Clean baselines are achieved by flushing for 2 minutes between sample runs. A typical image frame (shown after thresholding has been applied) from a proteinaceous sample is shown Figure 2. The system software provides measurement of area, equivalent circular diameter, perimeter, intensity, circularity, and maximum Feret’s diameter for each particle.

3.0 Performance Parameters of MFI

3.1 Sensitivity For Highly Transparent Particles

The results in Figure 3 are averaged from separate studies conducted by pharmaceutical laboratories on parenteral formulations containing proteinaceous particles. The solutions were analyzed using both traditional obscuration particle counters and MFI.
For particle size ranges relating to USP788, the measured concentrations for these sample types differ by one or more orders of magnitude and this difference becomes even greater for larger particles. The dramatically improved results provided by MFI were manually verified by direct observation of the images stored by the MFI system. Comparable experiments on transparent mineral particles have also demonstrated the higher sensitivity of the MFI technique. It can be hypothesized that the highly transparent particles are undersized and/or undetected by the obscuration instrument (due to a less sensitive threshold) resulting in these observed differences in measured concentration.

To illustrate the differences in transparency between PS beads and real proteinaceous particles, an experiment was performed to compare their respective measured intensities. MFI measures the ‘transparency’ of a particle by reading the intensity outputs of the pixels from the particle’s image on the digital camera. A higher pixel intensity value equates to a higher degree of transparency. The results in Figure 4 illustrate a clear difference between the observed MFI mean intensity for proteinaceous particles versus PS beads which are similar in size (10µm) and shape (as measured by circularity).

![Transmission: Proteinaceous Particles vs. PS Beads](image)

**Figure 4 - Transparency of Proteinaceous Particles vs. PS Beads**

### 3.2 Dependence on Particle Material Type

The direct, pixel-based imaging technique employed in MFI makes no assumptions of particle material type. Provided the presence of a particle results in sufficient contrast relative to the surrounding suspension fluid, the particle will be accurately sized. No calibration by the user is required.

In order to explore the material dependence of parameter measurements, MFI has been evaluated with unstained and stained PS beads and beads of borosilicate glass. The results in Figure 5 compare measurements of PS beads which were stained red and nearly transparent borosilicate glass beads (both nominally sized at 10µm). Despite the
widely different optical properties of the two types of beads, the sizing results are almost identical (note that these samples were not NIST-traceable).

This relative material-insensitivity demonstrates that MFI is well suited for the heterogeneous populations commonly found in intravenous solutions.

3.3 Sizing Accuracy and Repeatability

MFI contains design features which maximize sensitivity, accuracy and repeatability. These include an automatic, high sensitivity threshold setting, 10-bit threshold resolution and automatic background compensation for noise suppression. Experimental results with NIST-traceable PS spheres demonstrate the accuracy of MFI for sizing particles across its entire performance range of 0.75 to 400µm (see Figure 6).
3.4 Concentration Accuracy and Repeatability

MFI instruments provide sensitive detection, counting, and sizing of individual particles in each image frame. Since a frame represents a known sample volume, particle concentration can be directly measured. Figure 8 and Figure 9 highlight experimental results with NIST-traceable PS bead concentration standards (Duke Scientific Count-Cal) demonstrating the concentration accuracy of MFI. The manufacturer guarantees a specification of 3000 particles/ml with an accuracy of ±10% for both standards. The instrument was re-initialized between samples for a total of 6 runs on the 70µm standard and 3 runs on the 10µm standard.

Figure 8 - 10µm PSL Concentration Standard Accuracy
An additional benefit of MFI for concentration measurement is a higher upper limit compared to obscurcation instruments (~275,000 particles/ml vs. ~12,000 particles/ml respectively). This provides a correspondingly higher threshold before coincidence errors occur and reduces the requirement for sample dilution to obtain accurate results. Real-time images provided by MFI permit the user to immediately verify the likelihood of coincidence errors in each sample as well as determine the presence of potential sources of contamination. Figure 10 shows measurements of a 10-fold dilution series carried out with 10µm PS bead size standards.
3.5 Visible/Dense Particle Introduction

The stirring mechanism in the “top-down” gravity-assisted sample introduction system is designed to keep particles in a uniform suspension during testing. Results from a time study of a 70µm PS bead sample introduced with a 10ml syringe are shown in Figure 11. The data demonstrates that the concentration of the sample measured over a 15 minute period remains stable and settling is not a significant factor.

![Suspension Stability of 70µm PSDVB Spheres](image)

*Figure 11 - 70µm Concentration vs. Time*

3.6 Very Low Concentrations of Large Particles

An emerging requirement for parenteral drug analysis is to detect and measure very low concentrations of large (visible) particles in the presence of high concentrations of smaller particles. The source of these large particles can include contamination and formulation instability.

Table 1 and Table 2 are the results of experiments for the measurement of low concentration suspensions of NIST-traceable, 200µm PS beads. The first test used a concentration of ~20 particles per ml created by manually counting and suspending 110 particles into 5ml of filtered water. The second test used a concentration of 1 particle per ml created by mixing 5 particles into 5ml of filtered water.

<table>
<thead>
<tr>
<th>Parameter/Count per 5ml</th>
<th>R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>110</td>
</tr>
<tr>
<td>MFI Count (particles &gt;40µm)</td>
<td>92</td>
</tr>
<tr>
<td>Glassware Count (did not enter the system for analysis)</td>
<td>16</td>
</tr>
<tr>
<td>Image Verification Count (manual verification of stored images)</td>
<td>79</td>
</tr>
<tr>
<td>% Recovery - based upon Image Verification</td>
<td>72%</td>
</tr>
</tbody>
</table>

*Table 1 - Low Concentration Measurement (20 Particles/ ml)*
Table 2 - Low Concentration Measurement (1 Particle/ml)

Note 1: R1 and R2 contained additional particles which were shown by image analysis to result from contamination during sample preparation and handling.

Particles may be lost either by lodging in the glassware and tubing or by having passed through the flow cell outside the field of view where the frame is captured. These initial results demonstrate that MFI is capable of reliably detecting very low concentrations of large particles. They also demonstrate the importance of fluidics design, development of optimum protocols for sample introduction and glassware preparation. The value of stored image analysis in providing a method of verifying the analysis and diagnosing unexpected results is also demonstrated. Additional development is expected to further improve on current performance in “rare event” particle detection.

3.7 Shape Analysis and Particle Morphology

Because the light obscuration technique can only compare the signals received from real particles with those from PS spheres, particles are perceived as uniform spheres and particle size expressed in equivalent circular diameter. As was seen in Figure 2 and the images shown below, this assumption is misleading and particles vary widely in shape and uniformity.

In contrast to obscuration, MFI provides an image of each particle detected. These images are observed by the user and analyzed by the system software to provide quantitative information on particle morphology. Measurement parameters, which include Feret’s Diameter, area, perimeter, transparency and circularity, may be employed to create graphs and scatter plots which characterize the observed particle population.

As an example, MFI images shown below demonstrate the differences between ECD (equivalent circular diameter) and maximum Feret Diameter for various proteinaceous particles found in a sample of parenteral fluid. In this case, Feret’s Diameter is an effective parameter for distinguishing the particles based on their maximum dimension. Images on the left are the greyscale images as seen in the instrument while the images on the right are binary representations of the particles after thresholding.

<table>
<thead>
<tr>
<th>Parameter/ Count per 5ml</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
</tr>
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<tbody>
<tr>
<td>Sample</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Count (particles &gt;40µm)</td>
<td>7</td>
<td>26</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Image Verification Count</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>% Recovery (Image Verification)</td>
<td>100%</td>
<td>100%</td>
<td>60%</td>
<td>100%</td>
</tr>
</tbody>
</table>

ECD = 102.13µm
Max. Feret Diameter = 113.88µm

ECD = 120.88µm
Max. Feret Diameter = 237.88µm
4.0 Conclusions

Micro-Flow Imaging has distinct advantages over traditional obscuration counting in the analysis of sub-visible and visible particles in parenteral solutions. These include:

- Improved sensitivity and accuracy for near-transparent particle detection
- Results confirmation by direct image observation
- Insensitivity to particle material and shape
- Extended concentration range
- Ability to measure extremely low concentrations of outlier particles
- Additional information on particle morphology obtained by shape analysis
- The potential to isolate material types using morphological parameters

The combined image and data information provided by this new technology may be employed in a wide variety of applications in the parenteral development and manufacturing process stages. Examples include formulation development, stability assessment, contamination analysis, process control, quality control, diagnostics and troubleshooting.