International Journal of Pharmaceutics, 88 (1992) 303–312

IJP 02942

Validation of a computerized image analysis system for particle size determination Pharmaceutical applications

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(Received 6 March 1992)
(Modified version received 22 June 1992)
(Accepted 23 June 1992)

Key words: Image analysis; Validation; Particle size measurement

Summary

This study describes the methods used to validate the sample preparation procedure, measurement parameters, and statistical capabilities of an Olympus Cue-2 image analysis (IA) system The sample preparation of a micronized pharmaceutical compound for optical microscopy/IA was validated by scanning electron microscopy The effect of microscope and IA system configuration on measurement accuracy was demonstrated using National Bureau of Standards traceable particle-size standards. Magnification alone can induce a 20% error in the reported mean particle size of 10 μm diameter standard microspheres. By selecting the optimum microscope configuration, this source of error can be avoided. Aspect ratio (length to width ratio) measurements varied in accuracy from 4 to 450% depending on the true particle shape and orientation in the field of view. Algorithms employing Feret's diameter and area provided more accurate aspect ratio values. The IA system's statistical capabilities were validated by comparison to IA raw data reduction using Lotus 123® To meet regulatory guidelines, it is imperative that the entire system and analysis method be validated prior to routine use.

Introduction

Computerized image analysis (IA) systems provide the capability to observe the sample being analyzed, to consider particle shape factors during measurement, to discriminate between drug and excipient particles, and to remove operator bias during microscopic measurement. Image analysis has been employed to study particle size and quantitate the morphology of tabletting excipients (Prasad et al., 1987), to count and size particles in parenteral solutions (Tricome et al., 1986; Barber, 1988), and to determine the particle size of aerosols (Hallworth and Hamilton, 1976). Recent advances in computer technology have made IA systems more powerful, user-friendly, and affordable. For these reasons, image analysis has gained popularity as a versatile and reliable particle sizing technique. As the application of image analysis shifts from exploratory

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research to routine use in the pharmaceutical industry, the need for system validation arises (Guerra, 1988). Validation defines the system components and software, and demonstrates the system produces accurate results when operated according to standard procedures. Executing a rigorous validation protocol increases the user's understanding of the IA system's capabilities and limitations.

Generally, all IA systems operate by the same basic principles, but the parameters measured, algorithms for calculation, statistical capabilities, and file storage formats vary. The present paper describes the basic principles of an IA system, the backbone of an IA system validation protocol, and the techniques used to validate an Olympus Cue-2 computerized IA system, and recommends guidelines for validating IA particle sizing methods.

Basic principles of image analysis

Analog video images obtained from either an optical or electron microscope are input to a personal or mini-computer equipped with hardware and software capable of digitizing the incoming signal. The computer digitizes the analog signal by dividing the image into a matrix of picture points called 'pixels' (Inoue, 1986). Each pixel is assigned X, Y coordinates which describe its position in the matrix, and a grey-level value which corresponds to the image brightness at that position. The size of a pixel relative to the image is calibrated by comparison to a microscope stage micrometer or other fixed distance in the field of view. Discrete objects in the image are defined as contiguous pixels having a specific range of grey-level values. This grey-level range may be adjusted by the operator to achieve accurate object detection. The area of each object, minimum and maximum diameter, perimeter, aspect ratio, shape factor, etc., are calculated by the computer software. The data collected may then be tabulated, represented graphically, and/or stored in a software file.

The validation protocol

A validation protocol describes the steps which will be taken to determine whether the instrument functions as expected, identifies the person(s) responsible for each step, and defines the acceptance criteria for the tests conducted. An image analysis system validation protocol needs to address the following topics:

Hardware definition – identification of components, component layout, and circuit block diagrams.
Hardware specifications and operational limits.
Software definition – program names, versions, languages, hard-disk directory map, hard copies of configuration and auto-execution files.
IA program logic flow diagram.
Description of IA program error conditions and remedial action steps.
Test plan to validate data accuracy – parameter algorithms, input/output testing, stress limit testing, statistical capabilities, and acceptance criteria for tests conducted.
System security.
Change control procedures.
Operational procedures for instrument and peripherals.
Data archiving procedures.
Operator training.

Materials and Methods

Materials

The Olympus Cue-2 IA system consisted of the following hardware and software.

Hardware

Olympus BH-2 optical microscope with automatic stage movement and focusing *, Galai microscope controller *, Panasonic CCTV camera model WV-CD51 *, IBM PC-AT with RAM expansion, frame grabber, and VGA cards, and a mouse * (* Olympus Corp., Precision Instruments Division, Lake Success, NY); Amray 1200B scanning electron microscope (Amray Corp. Bedford, MA).

Software

Olympus Cue-2 Morphometry software, Version 3.01; Lotus 123 Version 2.01 (Lotus Development Corp., Cambridge, MA).

Standards

Certified diameter polystyrene-divinylbenzene (DVB) microspheres (Duke Scientific Corp., Palo Alto, CA); Calibrated microscope stage micrometer scale (Olympus Corp.).

The Olympus Cue-2 hardware configuration is shown schematically in Fig. 1. The Cue-2 Morphometry V3.01 software is a menu-driven IA program with image processing, measurement, and statistical capabilities. The operator may conduct analyses interactively using the mouse, or automatically by constructing a linkage of subroutines called an autoroute.

Methods

Hardware validation

Electrical and environmental specifications were obtained from the IA system and peripheral manufacturers. These parameters were monitored in the laboratory for a 2 week period prior to system installation.

Software validation

Measurement parameters Image analysis system measurement parameters are divided into *feature* parameters, e.g., particle area, diameter, aspect ratio, and *field* parameters, e.g., number of particles per field, field area/object area. For the purpose of particle size determination we are primarily concerned with the feature parameters. It is important to distinguish between the parameters measured by the system (independent) and those which are calculated from the measured parameters (dependent). Therefore, *feature* parameters are further classified as either independent or dependent.

The independent *feature* parameters (area, perimeter, and diameter) were validated by analyzing polystyrene DVB microspheres of certified diameter (National Bureau of Standards traceable). These standards were also used to determine the effect of microscope magnification on measurement accuracy. The microsphere standards, supplied as an aqueous suspension, were prepared for analysis according to the manufacturer's instructions as follows: (1) gently invert the bottle to disperse the particles; (2) immerse the bottle in an ultrasonic bath for 30 s; (3) immediately dispense the dispersion onto a microscope slide.

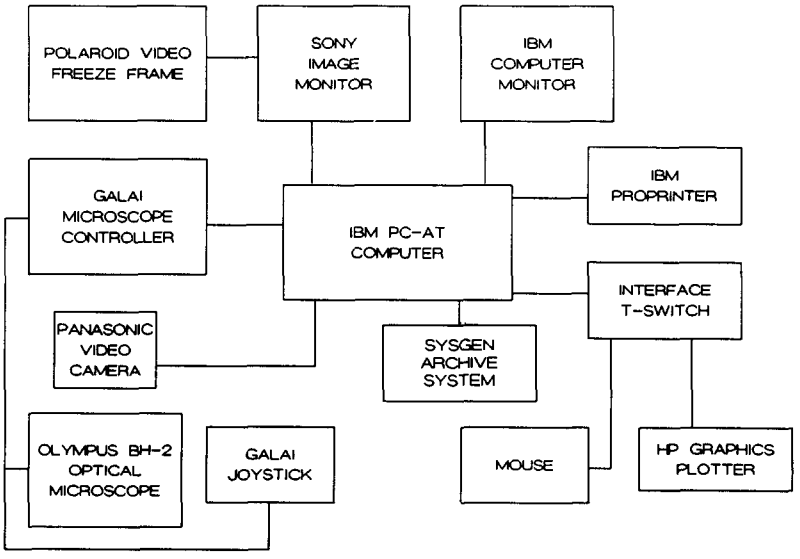

Fig. 1. Block diagram of Olympus Cue-2 image analysis system.

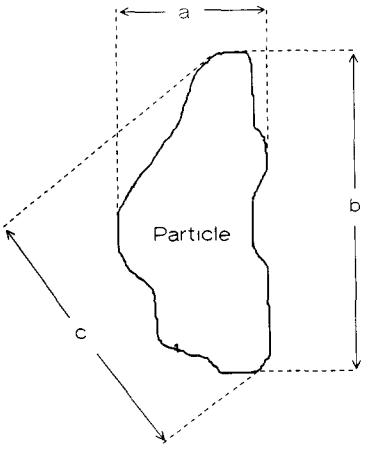

Fig 2. Caliper or Feret diameter measurement of a particle at various angles in the field of view. a, 0°, b, 90°; c, 225° (= 45°)

Certified standards such as microspheres are not sufficient for validating the IA system dependent parameters. Since many dependent parameters quantify a particle shape, peculiarities of the software algorithm which generate errors when measuring irregular particles may not be detected when validating with spherical standards. Furthermore, the software algorithm of a dependent parameter may differ from the parameter's commonplace definition. Therefore, the user must understand the IA system definition for each dependent parameter in order to validate the system. A dependent parameter must be validated using a standard, or calibrated object, which tests the parameter algorithm under stress limit (worst case) conditions.

For example, aspect ratio is commonly defined as the ratio of the minimum to maximum dimension of an object. IA systems typically define aspect ratio as the ratio of the minimum to maximum Feret diameter. A Feret diameter (Fig. 2) is a 'caliper' measurement of a particle at some angle θ in the X-Y plane (Joyce-Loebl, 1985). Although maximum Feret diameter measurement is highly accurate irrespective of particle orientation and number of Feret diameters measured (≥ 4), minimum Feret diameter accuracy is sensitive to these factors. To validate aspect ratio, the aspect ratio of a straight glass fiber was determined as a function of orientation and number of Feret diameters measured.

Statistical capabilities The statistical capabilities were validated by comparing the sample mean, standard deviation, and size distribution histogram generated by the Cue-2 with the evaluation of the IA raw data using another program, Lotus 123.

Program error conditions and remedial action steps The Cue-2 operator's manual provided a list of software error messages. To validate, conditions were created to prompt the error messages, thereby verifying their function in the software.

Remedial action steps are written procedures that describe what to do when program errors or hardware malfunctions occur. Suppose a power failure occurs during an analysis. Should the operator continue from where the analysis was interruped, or begin a new analysis? What should be done with the previously collected data? The system must be tested to determine what data is lost during an unintended shut-down, whether data may be added to a pre-existing file, and whether data is overwritten when a file is reopened. With this information, procedures may be written which are consistent with the laboratory policies for sample re-testing and disposition of analytical data.

Image analysis particle size method validation

IA method validation must address the sample preparation procedure, choice of dispersing medium, and image analyzer configuration, i.e., system magnification, image processing and data collection. The critical aspects of validating a given method will depend upon the sample being analyzed.

If the particle size distribution of the sample is very wide, e.g., a factor of 10^2, sampling technique and sample preparation will be critical to obtaining valid data. Selecting an appropriate combination of lenses to optimize magnification and resolution will be important for micronized particulates. Samples with complex morphologies may require specially formulated media for deaggregation, and more sophisticated definitions of particle size. Drug particles in suspension formu-

lations may be analyzed in situ, provided that the operator devises a means to distinguish the drug from particulate excipients in the formulation.

An optical microscopic/image analysis (OM/IA) method was developed to evaluate the size distribution of a micronized bulk powder. The OM/IA method was validated using an alternative sample preparation and imaging technique, scanning electron microscopy/image analysis (SEM/IA).

The purpose of independently measuring particles via SEM was to determine whether particles smaller than the resolution limit of optical microscopy, approx. 0.2 μm (McCrone and Delly, 1973), contribute significantly to the total volume of particles measured. The phrase 'contribute significantly' refers here to the relevance of the fines to the formulation. For example, detecting 1.0% fines below 0.2 μm in the formulation may not be critical to assessing safety or efficacy, whereas an assay detecting 1.0% of a harmful degradant might.

The bulk powder sample was prepared for SEM according to established procedures (Postek, 1980). The SEM was configured as follows: 2000 × magnification, 5 kV, 0° tilt angle. Representative photomicrographs were taken from 20 random fields of view. The photographic images of the particles were input to the image analyzer using the video camera mounted on a macro-viewing stand. A minimum of 500 particles were measured from the 20 photomicrographs.

Calculation of particle volumes from two-dimensional image analysis data requires approximating the magnitude of the particle's Z-axis. Spherical approximations of particle volumes from two-dimensional data tend to exaggerate the true particle volume. To obtain a better estimate of particle volume (V), an equation was derived which assumes that the particle maximum dimension (L) lies in the X-Y plane, and that the particle Z-axis (H) is approximately equal to the minimum particle dimension (W) in the X-Y plane. Given

$$V = L \times W \times H, \tag{1}$$

$$A = W \times L, \tag{2}$$

and

$$H \cong W; \tag{3}$$

then

$$V = L \times W^2 \tag{4}$$

and

$$W^2 = A^2/L^2 \tag{5}$$

therefore

$$V = (A^2 \times L)/L^2 \tag{6}$$

$$= A^2/L \tag{7}$$

or

$$V_i = \frac{A_i^2}{d_{i\,\max}} \tag{8}$$

where V_i represents the particle volume, A_i is the particle area and $d_{i\,\max}$ denotes the maximum Feret diameter, or length.

TABLE 1

Mean diameter of 9.87 μm certified polystyrene DVB microspheres measured at various magnifications

Objective lens magnification	Microscope magnification	Mean diameter (μm)	Uncertainty (μm)	RSD of distribution (%)	Error in mean diameter (%)
4×	12.5×	7.87	±4.12	22.3	20.3
20×	63.5×	9.94	±0.82	10.4	0.7
40×	125×	10.24	±0.41	9.7	3.7
100×	335×	10.87	±0.16	12.3	10.1

TABLE 2

Microscope configuration during analysis of certified standards

Standard identification (μm)	Objective lens magnification	Intermediate lens magnification	Ocular lens magnification	Microscope magnification	System calibration factor (μm/pixel)
1.09	100 ×	1 25 ×	2.5 ×	312 5 ×	0 164
7 0	40 ×	1 25 ×	5 ×	250 ×	1 655
9.87	20 ×	1.25 ×	5 ×	125 ×	0.423
24 7	20 ×	1.25 ×	5 ×	125 ×	0 423
49 7	10 ×	1.25 ×	5 ×	62.5 ×	0.837
102	10 ×	1 25 ×	2 5 ×	31 25 ×	1.655

A volume-weighted cumulative particle size distribution was compiled by summing the individual particle volumes using Eqn 9 (McCrone and Delly, 1973).

$$\% \text{ oversize} = \left[1 - \frac{\sum_{t=1}^{d} V_t}{V_t}\right] \times 100\% \qquad (9)$$

where d is the diameter in question.

These additional procedures were followed to ensure reproducible OM/IA sample preparation:

(1) A representative sample of bulk powder was obtained.
(2) The initial sample size (usually 1–5 g) was reduced to approx. 10 mg without introducing sample bias (Allen, 1981).
(3) The 10 mg sample was suspended in an appropriate liquid medium *.
(4) Harsh deaggregation techniques such as sonication and vortexing were avoided to prevent particle attrition. The vial was gently inverted several times to suspend the particles.
(5) A drop of the uniform suspension was transferred from vial to microscope slide using a pipet with an enlarged orifice, e.g., a serological pipet. This prevented seggregation of larger particles from the sample placed on the slide.

* Choice of the suspending liquid was based on the criteria that it provide sufficient wetting to separate aggregated particles and low refractive index liquid to produce adequate image contrast between the sample and medium Ideally, the sample should be insoluble in the suspending liquid to prevent changes in particle size during measurement The concentration of the sample in the liquid was adjusted to obtain a monolayer of non-contiguous particles on the glass slide.

Results and Discussion

Effect of magnification on measurement accuracy

When the particle size distribution of a sample is very wide, the magnification cannot be optimized for the entire sample distribution. To determine the types of errors which could occur when the microscope magnification is not optimum, 9.87 μm diameter standard spheres were measured at four different magnifications.

The size of a single pixel (IA system calibration value) and the resolution of the microscope objective determine measurement accuracy at low magnifications. At low magnification the error in reported mean diameter was approx. 20% (Table 1). Note that the uncertainty in measuring any single particle at low magnification (12.5 ×) was $\pm 50\%$ (± 4.12 μm).

High-magnification objectives typically have shallow depths of field. Consequently, not all particles in the measurement field are in focus simultaneously at high magnification. The contrast boundaries of poorly focused particles are

TABLE 3

Accuracy of particle size measurement: certified vs measured diameter of polystyrene DVB microsphere standards

Certified values			Measured values			Diameter measurement accuracy [c] (%)
Certified diameter (μm)	Uncertainty (μm)	RSD of distribution (%)	Measured diameter [a] (μm)	Uncertainty (μm)	RSD of distribution (%)	
1 09	N/A	0.75	1 05	±0.16	26.1	96
7.0	±0.2	5 7	7.5	±0 21	5.0	93
9 87	±0 06 [b]	0 8	10.4	±0 42	0.8	95
24.7	±0.7	5 7	26.1	±0.42	3.5	94
49.7	±1.0	6 3	52.1	±0.84	5.2	96
102	±2.0	5.4	103 0	±1 65	4 1	99

[a] Diameter = average of 8 Feret measurements.
[b] Uncertainty value obtained by particle array method (Duke and Layendecker, 1989).
[c] Measurement accuracy = measured diameter/certified diameter ×100%.

more diffuse and tend to exaggerate the measured particle size. At 335× magnification the measurement error was 10.1% for the 9.87 μm standard spheres (Table 1).

Validation of the independent parameters

The optimum measurement magnifications were identified for each of the certified standards. The microscope configuration used to analyze each standard is shown in Table 2. Table 3 compares the certified mean diameters of the polystyrene microsphere standards reported by the manufacturer (Duke Scientific Corp.) with the mean values determined by the image analyzer. The error values represent the width of a single pixel at the magnification used during the experiment. The accuracy of diameter measurement ranged from 93 to 99%, with a trend towards greater accuracy with the larger particle size standards (Table 3). The relative standard deviations reflect the spread of the sample size distribution.

TABLE 4

Accuracy of mean area [a] and perimeter [b] measurement using certified microsphere standards

Certified diameter (μm)	Measured mean diameter (μm)	Theoretical mean area (μm^2)	Measured mean area (μm^2)	Theoretical mean perimeter (μm)	Measured mean perimeter (μm)	Area measurement accuracy (%)	Perimeter measurement accuracy (%)
1 09	1.05	0.866	0.810	3.30	3.42	93.1	96.4
7.0	7.5	44.2	42.3	23.6	24.1	95.5	97.9
9.87	10.4	84.9	82.0	32.7	32 7	96.4	100
24.7	26.1	535.0	527.1	82.0	81.6	98.5	99.5
49.7	52.1	2132	2102	163.7	163.0	98.6	99.6
102	103	8332	8198	323.6	321.4	98.4	99.3

[a] Particle area, as defined by the IA system, i e., number of pixels per particle times the calibrated area of a single pixel
[b] Perimeter = number of boundary pixels in particle image times the calibrated length of a single pixel.
Theoretical mean area = π (measured mean diameter/2) ^{2}. Measured mean area = $(\Sigma_1^n A_i)/n$, where A_i is the measured area of particle i, and n denotes the total number of particles measured. Theoretical mean perimeter = π (measured mean diameter). Measured mean perimeter = $(\Sigma_1^n P_i)/n$, where P_i is the measured perimeter of particle i, and n total number of particles measured.

Particle area and perimeter measurement accuracy was also determined using the microsphere standards. Theoretical mean particle areas and perimeters were calculated from the measured mean diameter values (Table 4). These theoretical values were compared to the mean area and perimeter values measured by the image analyzer. Over the microsphere particle size range of 1.09–102 μm, the accuracy of area measurement ranged from 93–99%. Perimeter measurement accuracy was 96–99% over the same particle size range.

These data demonstrate that the IA system accurately measures the independent parameters area, perimeter, and diameter using spherical standards and optimum magnification.

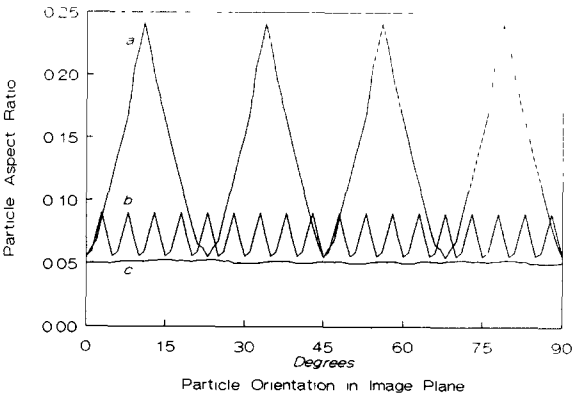

Fig. 3 Accuracy of particle aspect ratio measurement vs orientation in the field of view (a) 8 Feret system, (b) 36 Feret system, (c) A/d^2_{max}

Validation of the dependent parameters

The accuracy of aspect ratio measurement was determined by rotating a 0.05 aspect ratio straight glass fiber through 90° while taking measurements every 1°. Aspect ratio values between 0.05 and 0.25 were obtained using 8 Feret diameters (Fig. 3). Note that the measured aspect ratio diverged rapidly from the true value during rotation. Aspect ratio accuracy is related to the number of Feret diameters measured by the IA system and particle orientation in the field of view. Minimum Feret diameter measurement is most accurate when the particle's minor axis coincides with a Feret angle. Aspect ratio measurement error was less dramatic when measuring 36 Feret diameters because the probability of particle alignment with a Feret angle was greater. The time and memory space required to measure 36 Feret diameters per particle becomes prohibitive during routine analysis. Measuring 8 Feret diameters will suffice for most measurement purposes.

Aspect ratio measurement errors were minimized by employing an algorithm which produced accurate results irrespective of particle orientation. Accurate aspect ratio measurements were obtained by dividing the particle area by the square of the particle maximum Feret diameter (A/d^2_{max}). Fig. 3 shows results obtained using this algorithm. Accuracy of this derived algorithm, however, is dependent upon complete detection of particle area.

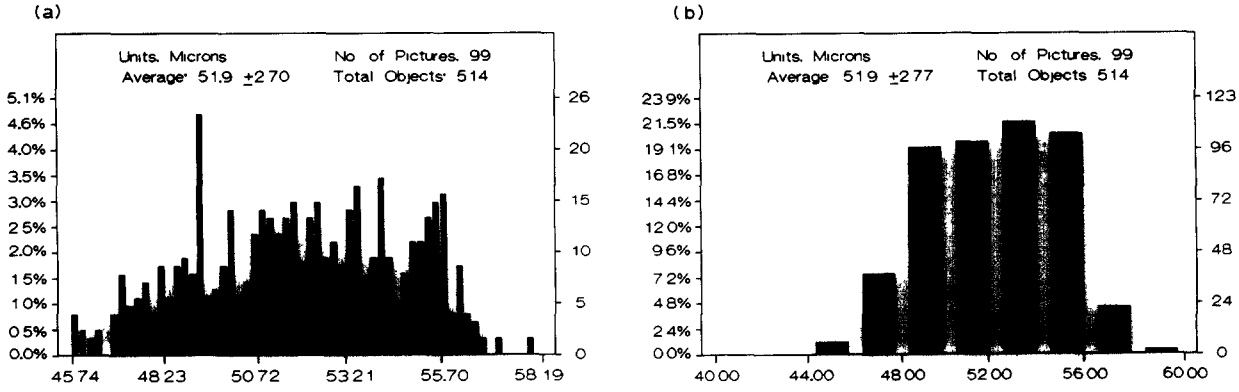

Fig. 4. Particle size histograms and statistical data generated by the Olympus Cue-2 Version 3.01. (a) Automatic scaling; (b) user-specified X-axis and number of histogram bins

Statistical capabilities

One limitation observed in a previous version of the Cue-2 statistical software package (Ver. 2.0) was the way in which the sample mean and standard deviation were calculated. To conserve memory space, the software calculated these values from the histogram rather than from the raw data collected. Consequently, modifying the *X*-axis scale of the distribution, or the number of histogram bins changed the reported mean and standard deviation of the sample. To circumvent this problem, the required statistical information was calculated from the image analyzer's ASCII-coded raw data using Lotus 123. This limitation of the software was corrected in Cue-2 version 3.01 (Fig. 4a vs b). Using the 49.7 μm certified diameter standard spheres, Cue-2 version 3.01 reported a mean diameter and standard deviation of 51.9 μm and 1.77 μm, respectively. Performing the same calculations using Lotus 123 and the ASCII data yielded 52.1 μm mean and 2.69 μm standard deviation. Differences between the Cue-2 and Lotus results were attributed to rounding error.

Particle size method validation

Measuring the micronized bulk drug via SEM/IA demonstrated that particles below 0.5 μm represent only 0.1% of the sample volume (Fig. 5). Hence, using the more convenient OM/IA technique and disregarding particles below 0.5 μm will not adversely affect the relevance of the experimental results.

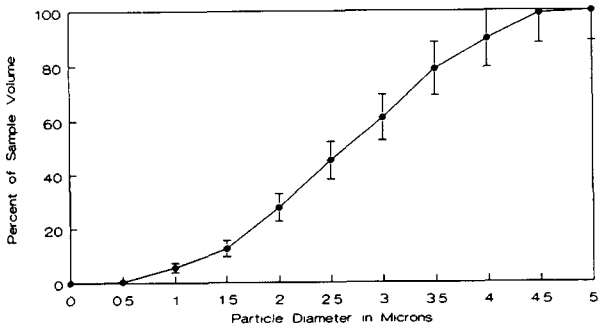

Fig 5 Volume weighted cumulative particle size distribution of a micronized bulk powder measured by the SEM/IA technique The error bars indicate that error is also cumulative

Inaccurate particle detection and/or agglomerated samples can invalidate an IA method. The guidelines for OM/IA sample preparation described in Materials and Methods facilitate completely automated analyses and increased experimental reproducibility. A well-dispersed sample minimizes the need for empirical computerized deagglomeration algorithms. High image contrast increases the precision of particle detection.

Conclusions

The measurement accuracy, parameter algorithms, and statistical capabilities of an Olympus Cue-2 image analysis system were validated using certified standards and Lotus 123. The independent measurement parameters were validated by comparison to certified polystyrene-DVB microsphere standards. Experiments demonstrated that measurement accuracy depends upon the magnification chosen. Higher magnification does not guarantee increased accuracy. At optimum magnification, measurement accuracy exceeded 93% for microsphere standards ranging from 1.0 to 100 μm nominal diameter. For samples with wide size distributions, choosing a magnification such that the largest particles barely fit in the image frame produced the most accurate results.

Investigations of the dependent parameter algorithm for aspect ratio revealed conditions which yield erroneous data. An alternative algorithm (A/d^2_{max}) measured the aspect ratio with increased accuracy, irrespective of particle orientation.

Discrepancies were discovered when comparing the statistical data generated by Cue-2 version 2.0 to raw data reduction using Lotus 123. This problem was corrected in version 3.01.

Optical microscopic/image analysis sample preparation procedures were developed to acquire reproducible data for particulate samples. An OM/IA method developed for a micronized bulk drug was validated by comparison to an alternative IA method employing an SEM. The guidelines provided for sample preparation and method development may be applied to suspension formulations, bulk powders with wide sample

distributions, and aerosol suspensions. Repetitive analyses and measurement by more precise or rigorous techniques are excellent means to validate a method.

Compared to measurement via manual microscopy, image analysis provides greater precision in quantitation, and increased speed through automated operation. Although these systems have become extremely refined in recent years, the potential for software errors should not be overlooked. These experiments demonsrate that an IA system can generate highly accurate and detailed particle size information, provided that the user understands the system's capabilities and limitations. Validated IA systems may be employed confidently for routine particle characterization within the pharmaceutical industry.

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