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Precision and accuracy of analysis of air-filled albumin microspheres using Coulter Multisizer Mark II

PER CHR. SONTUM* and CATH. CHRISTIANSEN

Research and Development Section, Nycomed Imaging AS, P.O. Box 4220 Torshov, N-0401 Oslo, Norway

Abstract: The suitability of the electrical sensing zone technique for routine analysis of air-filled albumin microspheres has been thoroughly investigated using three Coulter Multisizer Mark II instruments. The precision of the method, expressed as repeatability relative standard deviation (RSD), was found to be 1–2% for number distribution parameters and 3–4% for volume distribution parameters. Significant instrument-to-instrument variation as high as 11% was, however, also observed. Accuracy was evaluated from analyses of commercially available latex standards and from comparison of results from Coulter analysis with results from the following alternative techniques: light diffraction, optical microscopy and gravimetry. Accuracy, expressed as the difference from either certified values or values obtained with the alternative techniques, was found to be 100–106%.

Keywords: *Coulter Multisizer; electrical sensing zone technique; precision; accuracy; air-filled microspheres; albumin.*

Introduction

During the last few years an increasing need for well documented and well controlled techniques for analysis of particulate substances has developed within the pharmaceutical industry. Several analytical challenges have been identified, one of which is the routine quality control of new particulate drugs for parenteral use.

Albunex™, a new ultrasound contrast agent for medical imaging, is an example of such a drug. This product consists of a suspension of air-filled microspheres of heat aggregated albumin in a 5% (w/v) solution of human albumin. The microspheres are typically 1–15 μm in diameter and the thickness of the protein shell is about 15 nm [1]. The fraction of microspheres with diameters of 4.0–10.0 μm is assumed to give the main contribution to the acoustic backscatter after capillary filtration [2] and can be regarded as the active ingredient of the product.

To control the efficiency and safety of this product, an accurate and precise technique for characterization and quantitation of the air-filled microspheres is required. A natural choice of technique for resolving this analytical task is the electrical sensing zone method [3] which offers good resolution in the size range of interest. Results with the method, using three Coulter Multisizer Mark II instruments (Coulter Electronics Ltd, UK), are reported. Both instrument evaluation using commercially available latex standards and the results from validation of the analytical procedure for routine quality control of Albunex™ are presented.

Materials and Methods

Drug product

Albunex™ was produced by Nycomed Imaging AS, Oslo, Norway [4] (Albunex™ is a trademark of Molecular Biosystems Inc., San Diego, USA).

Instrumentation

Three Coulter Multisizer Mark II instruments, all fitted with a 50-μm orifice were used (Instruments I, II and III). The aperture current was fixed at 1600 μA, the gain factor was set at 2 and the siphon volume was fixed at 500 μl. Full range analyses were based upon a logarithmic diameter cumulative distribution;

* Author to whom correspondence should be addressed.

narrow-range analyses were based upon a linear diameter cumulative distribution.

Electrolyte

Isoton II (Coulter Electronics Ltd, UK) was chosen as the electrolyte. Isoton II is a phosphate buffered saline solution of NaCl (7.9 g l^{-1}), Na_2EDTA (0.4 g l^{-1}), KCl (0.4 g l^{-1}), NaH_2PO_4 (0.2 g l^{-1}), Na_2HPO_4 (1.9 g l^{-1}) and NaF (0.3 g l^{-1}) in water. The solution was passed through a 0.22-μm filter (Millipak 40, Millipore Corp., USA) and equilibrated to 27.0 ± 0.5°C prior to use.

Instrument calibration

Each Coulter Multisizer instrument was initially calibrated according to the manufacturer's recommendation with a 5-μm latex calibration standard (Coulter Electronics Ltd, UK). Owing to small differences in actual aperture diameter, the three instruments gave different calibration factors (K_d), ranging from 490–564. As a result, the actual full measuring range for the three instruments differed: Instrument I, 1.23–38.27 μm; Instrument II, 1.07–33.25 μm; and Instrument III, 1.09–33.93 μm. After the initial calibration, the calibration factor was regularly controlled but not changed as long as the variation was within the inherent random variation of the calibration procedure (RSD typically ± 1%). All reported results were obtained with the initial calibration factor for each instrument.

Instrument evaluation

Latex standards were used to evaluate the Coulter Multisizer Mark II instruments with respect to precision and accuracy. The precision was determined using monosized 5-μm polymer latex (SS-051-P, Dyno Particles, Norway). The latex preparation was diluted 10-fold in 0.15% (w/v) sodium dodecylsulphate (BDH Chemicals, UK) and 500-μl aliquots of the diluted suspension were stored in tightly capped glass vials. A single sample from this batch of diluted latex suspension was analysed on each of 20 operative days using a sample volume of 200 μl and an electrolyte volume of 200 ml. Analyses were performed in the full range with 64 channels. As analytical response parameters the number concentration of particles per ml sample and the arithmetic number mean diameter, D(1,0) (μm) [5], were calculated. Before each analysis of sample, the background counts in the pure Isoton II were measured.

To validate the accuracy of the latex analysis, four different monosized latex suspensions (Duke Scientific Corp., USA) with certified NIST/NBS traceable mean diameters, were analysed in triplicate on each instrument. Nominal standard sizes investigated were 2.0, 4.0, 10 and 25 μm (Duke Catalogue no. 4202A, 4204A, 4210A and 4225A, respectively). Sample volumes for the Coulter analysis ranged from 25 to 1500 μl and electrolyte volumes ranged from 100 to 200 ml, depending on the particle concentration of each standard. Analyses were performed with 64 channels in the narrow range, set to the nominal latex diameter ±25%. As response parameter, the arithmetic number mean diameter, D(1,0) (μm) was calculated.

Validation of the Coulter analysis of air-filled microspheres in Albunex™

In the standard procedure for analysing Albunex™ with a Coulter Multisizer Mark II, a homogenous sample of 20 μl Albunex™ was added to 200 ml of electrolyte and the analysis was performed in the full range with 64 channels. Each analysis was performed in triplicate. The analytical response parameters routinely reported were: total number concentration of microspheres per ml suspension; number concentration of microspheres with diameters from 4.0 to 10.0 μm per ml suspension; number concentration of microspheres equal to or larger than 10.0 μm per ml suspension; number concentration of microspheres equal to or larger than 25.0 μm per ml suspension; number mean diameter, D(1,0) (μm); and volume concentration of microsphere in per cent of suspension volume.

For validation of intra-instrument precision, ten samples from each of three batches of Albunex™ were analysed on a single Multisizer instrument. Analyses were performed by three trained operators during a 4-week period. Precision was calculated as pooled (cross-sample mean) standard deviation (SD) and relative standard variation (RSD) for each routinely reported response parameter.

To evaluate inter-instrument precision, six samples from one batch of Albunex™ were analysed on each of three instruments and in a randomized pattern. The difference between results from each instrument was calculated for each sample. The significance of the average

difference was tested with a paired two-tailed *t*-test. Differences were considered significant if the calculated probability level, *P*, was greater than 95%. As an additional response parameter for this evaluation, the number concentration of microspheres of 4.0–5.0 μm per ml suspension was calculated.

Validation of the method accuracy was performed by comparison with alternative techniques. Comparisons were made between results from Coulter counting and the following alternative techniques; optical microscopy, light diffraction and gravimetric analysis. To make comparison possible, two additional Coulter responses, volume mean diameter, D(4,3) (μm) [5] and the fraction of the number distribution equal to or less than 10.0 μm (%) were calculated. Details regarding the alternative methods are given below.

The volume concentration of microspheres was determined gravimetrically by measuring the density of the product. Assuming all microspheres to be air-filled and the surrounding shell of protein to contain a negligible mass, the total volume concentration of air in the product may be calculated as

$$\text{Volume concentration (\%, ml/ml)} = (1 - \rho_{\text{Albunex}^{TM}}/\rho_{5\%\,\text{HSA}}) \cdot 100$$

where $\rho_{\text{Albunex}^{TM}}$ and $\rho_{5\%\,\text{HSA}}$ are the densities of Albunex™ and human albumin solution 5%, respectively. Tripicate analysis on each sample was performed by weighing 500-μl samples with a Mettler AT 261 balance (Mettler–Toledo A.G., Switzerland) and calculating the density of Albunex™ (g ml^{-1}) as twice the measured weight. The density of human albumin solution 5% was set equal to 1.01 g ml^{-1}.

Microsphere number mean diameter, (D(1,0) (μm) and the percentage of microspheres with diameter equal to or less than 10.0 μm were determined by optical microscopy. Microscopic examination of the Albunex™ microspheres was performed with a Nikon Labophot microscope (1.25×) (Nikon Corp., Japan) with a 10× ocular and a 100× objective fitted with a Nikon 3WA camera. Approximately 10 μl of undiluted sample was applied to a plain glass slide, covered with a glass cover slip and placed under the microscope. Four photographs of the sample were taken from various but representative parts of the slide. The diameter of all microspheres (some 200 in each photograph) was measured by sliding a transparent ruler across each photograph and counting the number of microspheres with diameters 1, 2, 3, . . . 20 μm. To calibrate the actual optical magnification, a monosized 5.0-μm polymer latex standard (SS-051-P, Dyno Particles, Norway) was measured using the same procedure as for the Albunex™ sample and microsphere diameters were calculated using the found calibration factor. Responses were calculated as the mean and SD cross-calculated D(1,0) and % <10.0 μm from each photograph.

The volume mean diameter D(4,3) (μm) and volume concentration of microspheres in percentage of suspension volume were determined by light diffraction on a Malvern Mastersizer 1002 (Malvern Instruments Ltd, UK). Measurements were performed in a small volume sample cell (MS-1) using a 200-μl sample in 100 ml of Isoton II. A single analysis was performed on each sample. Results were calculated using a model independent algorithm with all experimental data points included. This model does not assume any specific shape of the size distribution, but is a non-constrained best fit to experimental data. The optical presentation which describes the refractive index difference between the particles and the suspending media and also the ability of the particles to absorb light, was set equal to 1400. Such a presentation should correspond roughly to air bubbles in water and should therefore be a good approximation of Albunex™.

Results and Discussion

Instrument evaluation

The results from analysis of a suspension of 5.0-μm polymer particles on 20 different occasions using three different Coulter Multisizers are given in Table 1. A satisfactory intra-instrument reproducibility was obtained for both number concentration and number mean diameter on each instrument. The inter-instrument reproducibility was, however, less acceptable with a maximum deviation of about 10% for the number concentration. Approximately half this difference was contained in the latex peak (4–6 μm region) while the other half arose from different amounts of noise detected in the lower channels. The differences in detected noise also caused the observed differences in the number mean diameter.

Table 1
The reproducibility of the number concentration and the number mean diameter for a 5.0-μm polymer suspension analysed on three Coulter Multisizers

	Number conc. (10^7 ml^{-1})*		Number mean diam. (μm)*	
Instrument no.	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
I	1.61 ± 0.03	1.8	4.99 ± 0.03	0.6
II	1.79 ± 0.03	1.7	4.89 ± 0.02	0.2
III	1.76 ± 0.02	1.1	4.88 ± 0.02	0.4

*Mean ± SD (n = 20).

Table 2
Certified and measured number mean diameters for four different latex standards analysed on three Coulter Multisizers

Nominal diameter (μm)	Certified number mean diameter (μm) (uncertainty in %)*	Measured number mean diameter in % of certified value†		
		Instr. I	Instr. II	Instr. III
2.0	2.06 (1.2)	101.4 ± 0.0	103.3 ± 0.0	100.0 ± 0.6
4.0	3.98 (0.8)	101.7 ± 0.3	102.9 ± 0.4	100.4 ± 0.0
10	9.87 (0.6)	1005. ± 0.1	103.7 ± 0.2	101.4 ± 0.2
25	25.7 (0.8)	104.8 ± 0.0	104.7 ± 0.0	103.5 ± 0.0

*Uncertainty is certification range, i.e. true mean diameter is certified to be mean diameter ± stated uncertainty.
†Mean ± SD (n = 3).

The results from analysis of four different latex standards with NIST/NBS certified number mean diameter are listed in Table 2. Three out of 12 determinations of the number mean diameter were within the certified range. The remaining nine determinations were within ±5% of the certified value. The observed deviations are regarded as quite acceptable when taking into account the rather subjective procedure for initial calibration of the instruments.

Factors affecting the reliability of results from the Coulter analysis of Albunex™

For reliable analysis of the air-filled Albunex™ microspheres by Coulter counting, two method parameters were found to be of main importance. These were the temperature of the electrolyte solution and the sample volume.

As seen from Fig. 1, a sharp decrease in the detected number concentration and number mean diameter was observed when the temperature of the Isoton II solution decreased below 20°C. The effect is due to undersaturation of air in the electrolyte when it is cooled down. This causes a diffusion of air from the microspheres to the surrounding electrolyte. To ascertain that the electrolyte solution was over-saturated with air, a temperature well above the electrolyte storage temperature was specified.

It was also shown that the results from Coulter analysis were dependent on the volume of Albunex™ analysed. As the sample volume increased, the measured number concentration decreased while the mean diameter increased. The effect may be due to an increased probability of coincident passage of two or more microspheres through the aperture, leading to fewer counts of larger microspheres. The consequent dependency of results on actual sample concentration was minimal and negligible by choosing a small sample volume of 20 μl.

Suitability of the selected measuring range

The content of microspheres with diameters above the upper detection limit of the 50 μm aperture (30 μm) was investivated using a Multisizer equipped with a 140-μm aperture which extends the measuring range to about 84 μm. Results from analysis on three batches of Albunex™ showed that typically 0.01% of the microspheres had diameters above 30 μm and typically 0.001% had diameters above 50 μm. These results were qualitatively confirmed by optical microscopy. None of more than 40,000 visually sized microspheres were found to be larger than 30 μm. Consequently, the selected 50-μm aperture is regarded as suitable for the complete characterization of the microspheres in Albunex™.

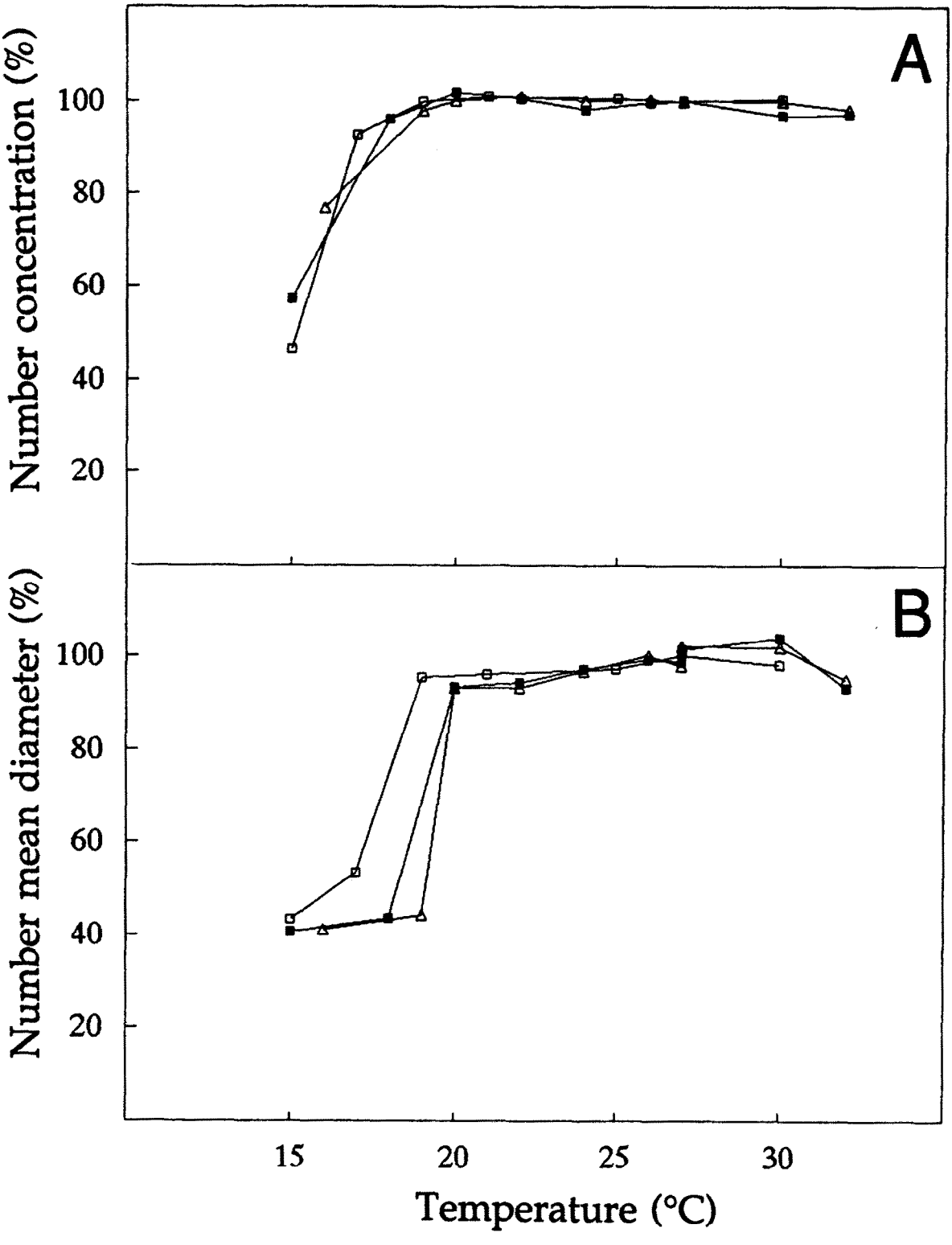

Figure 1
Effect of temperature of the Isoton II solution on the measured microsphere number concentration (A) and number mean diameter (B). Results reported in percentage of value at 27°C, are given for three different Albunex™ samples.

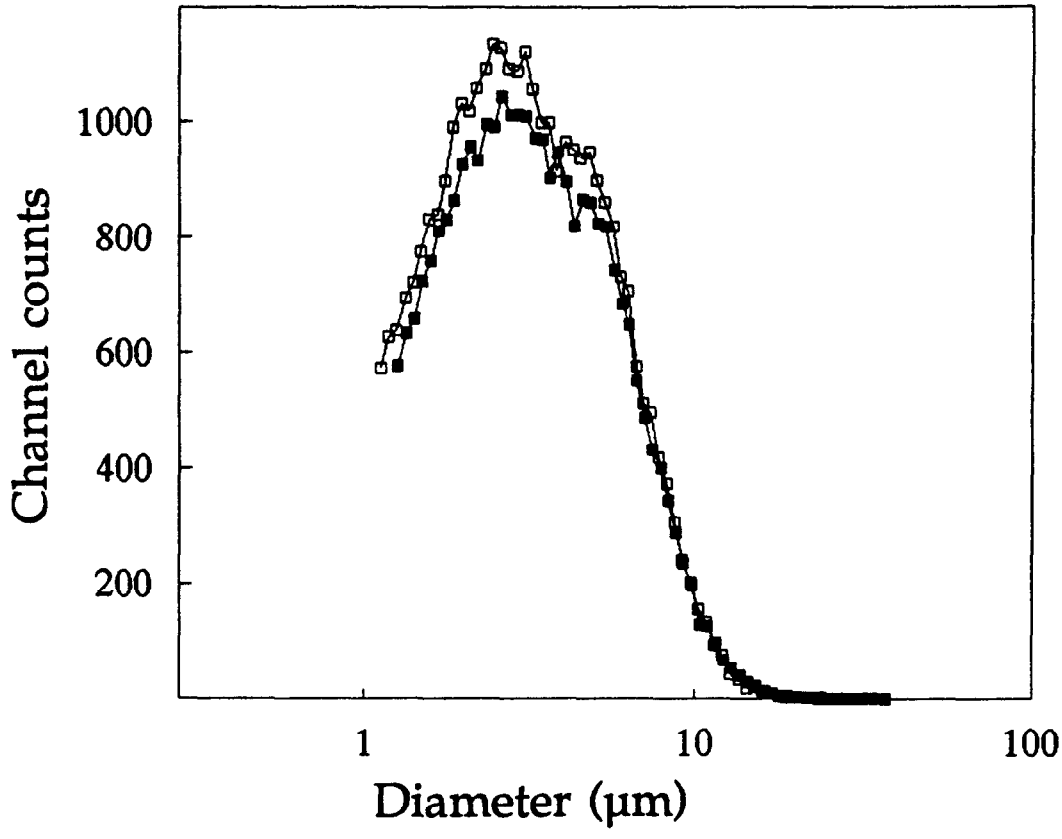

Figure 2
The number distribution of a single Albunex™ sample measured on two different Coulter Multisizer instruments (Instrument I and II). Each distribution is the average of triplicate analyses.

Table 3
Repeatability of reported Albunex™ parameters expressed as pooled SD and RSD from triplicate analysis on 30 samples

Parameter	Mean	SD	RSD (%)
Number concentration, 4.0 to 10.0 μm (10^8 ml^{-1})	2.13	0.03	1.3
Number concentration, ≥10.0 μm (10^8 ml^{-1})	0.29	0.004	1.3
Number concentration, ≥25.0 μm (10^6 ml^{-1})	0.41	0.11	26
Number concentration, 1 to 30 μm (10^8 ml^{-1})	7.48	0.09	1.3
Volume concentration, 1 to 30 μm (%, v/v)	7.5	0.25	3.4
Number mean diameter, 1 to 30 μm (μm)	3.77	0.02	0.6

Table 4
Mean observed difference between instruments I, II and III for some reported Albunex™ parameters

Parameter	Instrument I Mean	Mean instrument difference ±95% confidence interval*	
		I − II	I − III
Number concentration, 4.0–5.0 μm (10^8 ml^{-1})	0.69	−0.033 ± 0.016	−0.058 ± 0.012
Number concentration, 4.0–10.0 μm (10^8 ml^{-1})	1.96	−0.072 ± 0.029	−0.093 ± 0.039
Number concentration, 1–30 μm (10^8 ml^{-1})	5.97	−0.593 ± 0.055	−0.628 ± 0.067
Number mean diameter, 1–30 μm (μm)	3.79	0.147 ± 0.013	0.152 ± 0.015

*Triplicate analysis of six samples on each instrument.

Validation of the Coulter analysis of Albunex™

The routine Coulter analysis of Albunex™ was validated for intra-instrument precision by analysing 30 samples of Albunex™ from one production batch in triplicate using Instrument I. The results are summarized in Table 3. Taking into account the very broad size distribution of fast floating microspheres in the samples analysed, the results demonstrate an acceptable repeatability. The high RSD for the number concentration larger than 25.0 μm is due to the very few microspheres with diameters above this size, causing a relative large statistical variation in their quantification. The precision for the other parameters was comparable to the precision obtained when analysing latex samples.

Inter-instrumental precision was validated by comparison of results for six samples of Albunex™ analysed in triplicate on each of the three instruments. The typical number distribution obtained with two of the instruments is illustrated in Fig. 2. In Table 4 are listed the results for response parameters where significant (>95%) differences were observed between the instruments.

For some of the reported parameters such as the total number concentration and mean diameter, the instrument-to-instrument variation is partly caused by a difference in the measuring range for the different instruments. In addition, the counting efficiency for the different regions of the measuring range is instrument dependent. As a result, the relative average difference in the number concentration between 4.0–10.0 μm was as high as 5%. For other comparable parameters in the lower end of the measuring range, such as the number concentration between 4.0–5.0 μm, differences for single samples of up to 11% were observed.

Validation of the method accuracy was complicated by the fact that no reference substance exists which can be used for spiking purposes. As commercial number concentration standards are also unavailable, validation of method accuracy had to rest on comparison with alternative techniques. Comparison was made between the results from Coulter counting and the following alternative methods: optical microscopy, light diffraction and gravimetric determination of the volume concentration of air-filled particles. It is neither claimed nor validated that either of these techniques are more accurate than the Coulter Multisizer.

Samples from one Albunex™ batch were analysed by both optical microscopy and Coulter analysis, and results for the number mean diameter (D(1,0)) and fraction less than 10.0 μm were compared. Also, the volume mean diameter (D(4,3)) and the volume concentration of microspheres in four Albunex™ samples were determined by light diffraction and compared to Coulter analysis on the same samples. Finally, the volume concentration of nine samples from three different batches of Albunex was determined gravimetrically and the results were compared with Coulter analysis on the same samples. The results from these analyses are summarized in Table 5 and illustrated in Figs 3 and 4.

The results from the various analyses agreed quite well. The Coulter analysis yielded results which were negligibly different from those obtained by optical microscopy. The measured volume concentration of microspheres as determined by Coulter analysis also agreed within experimental error with light diffraction analysis and gravimetric analysis. The only significant difference found was a 6% lower volume mean diameter by light diffraction than by Coulter analysis. In view of the fundamental differences between these two techniques, this correlation is regarded as rather good.

Table 5
Comparison of size distribution parameters of Albunex™ as determined by Coulter Multisizer analysis and various alternative techniques

Parameter	Alternative technique	Coulter result	Alternative technique result	Mean difference ±95% confidence interval
Number mean diameter, D(1.0) (μm)	Optical microscopy	3.70	3.7	0
Percentage LT 10.0 μm, (%)	Optical microscopy	96	98	2
Volume mean diameter, D(4.3) (μm)	Light diffraction	10.9	10.2	−0.7 ± 0.1
Volume concentration, (%(v/v))	Light diffraction	6.1	6.2	0.1 ± 0.6
Volume concentration, (%(v/v))	Gravimetric analysis	7.5	7.4	−0.1 ± 0.3

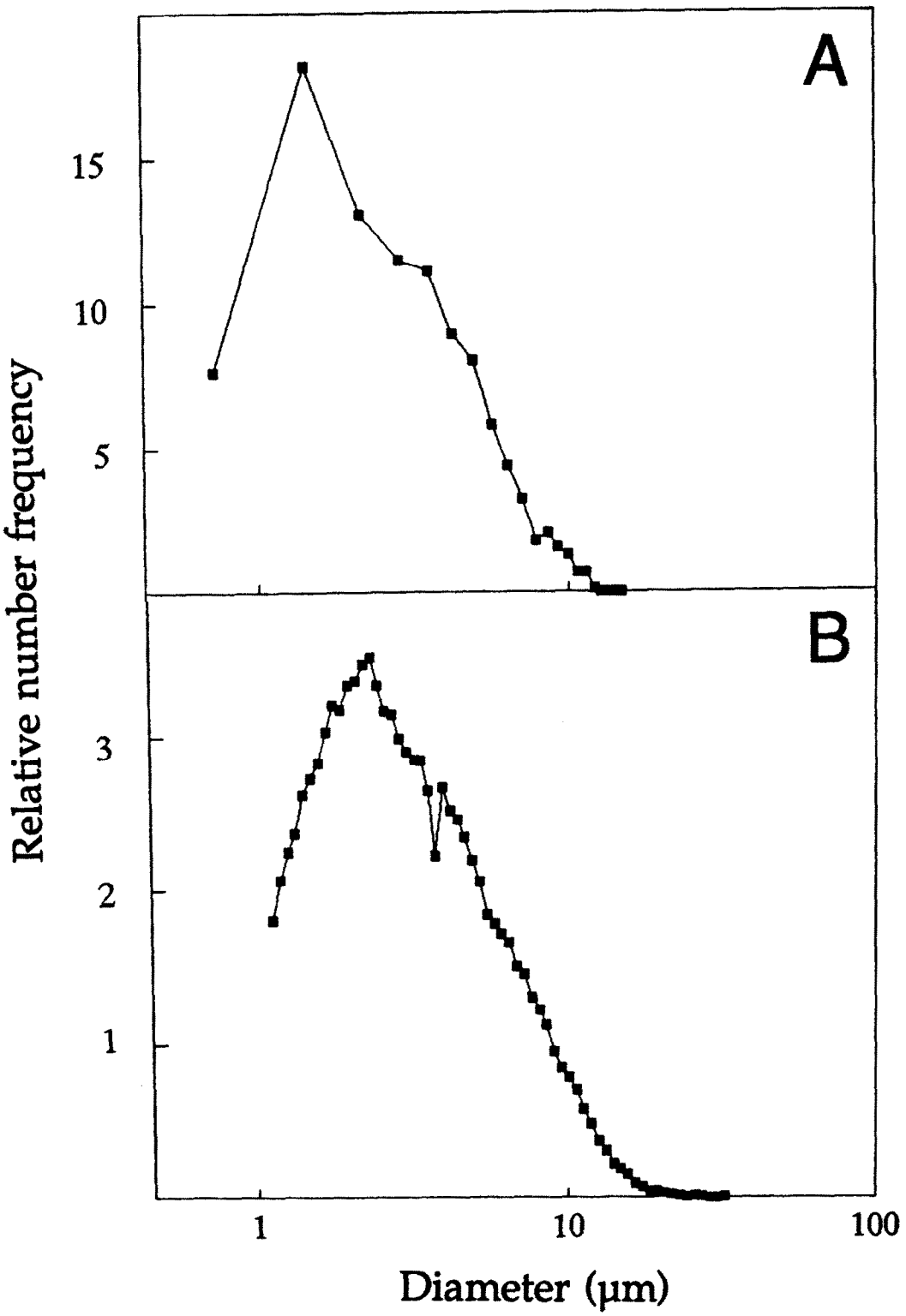

Figure 3
The number distribution of microspheres in an Albunex™ sample measured with optical microscopy (A) and with the Coulter Multisizer (B).

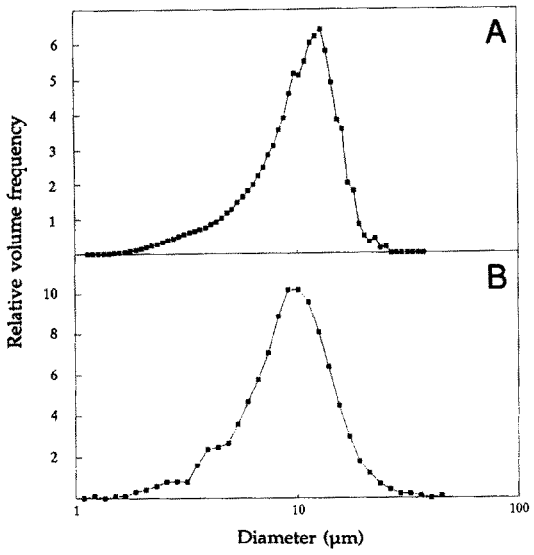

Figure 4
The volume distribution of mcirospheres in an Albunex™ sample measured with the Coulter Multisizer (A) and with the Malvern Mastersizer 1002 (B).

Conclusions

The electrical sensing zone technique is capable of delivering analytical results of a quality acceptable for routine quality control in the pharmaceutical industry.

Precision, expressed as repeatability relative standard deviation, is typically 1–2% for parameters derived from the detected number distribution and 3–4% for parameters derived from the volume distribution. However, for some analytical parameters, particularly those concerning the lower size range, instrument-to-instrument variations greater than 10% were observed. This effect may well lead to the necessity of using correlation factors to compare results from different instruments.

The accuracy of the Coulter analysis was documented to be in the 98–106% range when compared either with certified values for standards or with the results from analysis of Albunex™ by three alternative techniques: optical microscopy, light diffraction analysis and gravimetric analysis.

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References

[1] C. Christiansen, H. Kryvi, P.C. Sontum and T. Skotland, *Biotechnol. Appl. Biochem.* **19**, 307–320 (1994).
[2] L. Hoff, C. Christiansen and T. Skotland, *J. Ultrasound Med.*, in press (1994).
[3] T. Allen, in *Particle Size Measurements* (T. Allen, Ed.), pp. 392–413. Chapman and Hall, London (1981).
[4] D. Cerney, C. Vista, G.J. Mills and P.J. Westkaemper, U.S. Patent 4, 957, 656 (18 September 1990).
[5] H. Heywood, *J. Pharm. Pharmacol.*, suppl. 15, 56 Y (1963).

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