Rapid Publications
Examination of Insulin Formulations Using Quasi-Elastic Light Scattering

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SUMMARY

A selection of neutral insulin formulations from commercially available vials was examined by quasi-elastic light scattering. In preliminary investigation, significant differences in the particulate nature of insulins from different manufacturers were apparent. Some preparations contained only small diameter species believed to be insulin hexamer while others showed evidence of larger aggregates and microcrystals. Addition of small amounts of bicarbonate had no detectable effect on the small species but dissolved the microcrystals. Speculation on further and more quantitative use of this new technique for analysis of insulin solutions is reported. DIABETES 31:364–366, April 1982.

The aggregational behavior of pork and beef insulins in both acid and neutral formulations has been widely reported in the literature.[1–7] Because of this tendency of insulin to associate in both neutral and acid solutions, there has been some speculation as to which form is biologically active (i.e., whether it is the monomer or higher polymers which interact with the receptor.)[8] Goldman and Carpenter[3] and Pekar and Frank[4] claim that the dimer and higher polymers should not exist at physiologic concentrations of the hormone. More recent evidence[8,9] has indicated that the monomer is the active form and that the same hydrophobic region of the B chain is responsible both for dimer formation and for receptor-monomer binding.[9–11] It seems that the dimer and any higher polymers present must be dissociated prior to having hormonal action.

In this preliminary investigation a study of particle sizes present in a selection of commercial insulin preparations was undertaken using the quasi-elastic light scattering technique. This novel technique is capable of examining insulin formulations in situ without the dilution, the addition of buffers, or the other maneuvers usually used to examine peptide containing solutions. Such an analysis is of interest because of the active form question raised earlier and also because of problems caused by aggregates obstructing insulin infusion devices.[5,12]

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Received for publication 11 January 1982.

MATERIALS AND METHODS

Determination of particle size. Quasi-electric light scattering allows the measurement of diffusion coefficient rapidly and with considerable precision. The method has been fully discussed elsewhere.[13,14] The particular device used in this research employed a thermally jacketed scattering chamber, a helium-neon laser (Model HN-15 Jodon Engineering Associates Inc., Ann Arbor, Michigan), a quantum photometer (Model 1140, Princeton Applied Research, Princeton, New Jersey) and a 64-channel autocorrelator (Langley-Ford Instruments, Amherst, Massachusetts). Analysis of the resulting digital autocorrelation function was performed on an on-line NOVA-2 minicomputer (Data General Inc., Southboro, Massachusetts) using two different methods, the method of cumulants described by Koppel[15] and Kendall and Stuart[16] and a least squares fit to a sum of two exponentials.

In the cumulants method, one obtains an estimate of the average correlation time. From this, the average hydrodynamic diameter of a distribution of particles can be computed when the sample temperature, the refractive index of the solution, the wavelength of the laser light (632.8 nm), and the scattering angle are known.

This computation provides reasonable estimates of the average hydrodynamic diameter for spherically shaped particles or for ellipsoids or rods whose major axis is about 10% of the wavelength. In these nonspherical systems, the average hydrodynamic diameter corresponds to the Stokes radius of the particle. Some indication of the width of the size distribution of scattering particles can be obtained from the second cumulant.

Many of the insulin formulations clearly have two or more

components. In these cases, the sum of two or more exponentials provides better fits, as judged by sums of squares, than the cumulants method. A fitting function is therefore used to distribute the observed electric field autocorrelation function, according to the relative amplitudes of terms describing the average correlation times estimated for each component.

In all the measurements reported here, the sample was temperature controlled at 24.3°C. The scattering angle for all experiments was 90°.

Insulin samples. Vials of neutral insulins from four manufacturers (Lilly, Novo, Nordisk, and Connaught) were purchased from local distributors. All samples were within their recommended shelf life with expiration dates ranging from 12 to 24 mo. The vials were opened and 1–2-ml aliquots of insulin transferred into 2-ml capacity break-seal vials (Wheaton Glass Co., New Jersey) using either precleaned disposable pasteur pipets or Oxford propipets fitted with precleaned polypropylene tips. The pipets and tips were washed with distilled water and dried by rinsing with acetone. Contact of the insulin with metals and subjection to shear forces was not desired so syringes were not used. The break-seal vials were prepared by washing with chromic acid, rinsing with distilled water, and drying in a horizontal position in an oven. The filled vials were quickly heat sealed, care being taken not to heat more than the top of the long vial neck (which had not been exposed to the insulin solution) and to keep the solution away from the upper heated portion of the glass until cooling had occurred. This method was designed to minimize external contamination, dust accumulation, and denaturation of the protein.

Samples containing bicarbonate were prepared as follows: Sodium bicarbonate (Fisher Chemical Co.) was dissolved in water through which CO_2 was being bubbled as dissolution occurred. The pH was then adjusted to 7.4 and the solution sealed in a vial under a CO_2 atmosphere. The stock solution was never stored for more than 2 h prior to use. A 100-μl aliquot of the stock bicarbonate solution was added directly to 2 ml of insulin formulations, and the sample vial sealed immediately. This procedure was to prevent CO_2 loss, pH change, or bicarbonate depletion.

RESULTS

The results of this study are presented in basically a qualitative form. As will be discussed below, several control experiments must be performed and fitting methods optimized before fully quantitative results can be reported.

In Table 1, the major characteristics of the sample groups tested are shown. There are clearly significant differences in the gross characteristics of the manufacturers' products. Electric field autocorrelation functions from samples from manufacturers C and D fell to background after only 5 or 6 channels (channel width = 20 μs). Count rates from these samples were also quite low, indicating the presence of uniformly small particles. The small particle size of the scattering species approached the resolution limit of the spectrometer so that the particle size quoted (10 nm) is considered to be an upper limit.

The replicates of manufacturers samples were in good agreement with each other. One vial from each was sampled in triplicate to estimate discrepancies incurred by transfer and handling of the insulins.

TABLE 1
Characteristics of neutral insulin formulations as determined by quasi-elastic light scattering

Manufacturer	Type	No. of samples	Average hydrodynamic diameter (nm)	Comments
A	Pork	8	100–1,000	All samples contained two or more components
B	Beef & pork	6	>10,000	All samples contained many large particles with considerable specular reflection. Particles were likely microcrystals.
	Pork	6	>10,000	
C	Pork	5	≤10	All samples
D	Pork	7	≤10	All samples

Table 1 does not include results from samples in which the protein might have been overheated (denatured) during the sealing procedure. A few such samples were studied, however, and an enormous increase in particle size was apparent. Also, previously opened vials from manufacturer C or ones from which samples were transferred using a syringe and needle showed particles 10–25 times larger than in others not subjected to these maneuvers.

The results of the bicarbonate-treated samples are not included in Table 1. Since only one vial of each insulin type was treated in this manner only preliminary statements can be made. Bicarbonate does not appear to markedly affect the average aggregate size in the solution. It does, however, remove the microcrystalline species that lead to the specular reflections. Hence there appears to be a fundamental distinction between "aggregates" and "microcrystals" in these preparations.

DISCUSSION

Quasi-elastic laser light scattering is a noninvasive technique which has allowed the examination of particle size in essentially native, commercially available insulin formulations. The results presented indicate that the different manufacturing processes used produce qualitatively different insulin formulations. No judgment can be made at this stage as to whether small homogeneous particle size is particularly advantageous to the diabetic patient. Further studies in conjunction with clinical trials may answer this question. It is interesting that the bicarbonate ion does not appear to affect the particle distribution, although in infusion devices it delays the type of aggregation[17] that in due course obstructs the delivery catheters.

To estimate the extent of polymerization in these solutions, the particle size must be related to the dimensions for the species which have been obtained by X-ray crystallography. Blundell et al.[18] describe the dimer as oval, about 40Å (4 nm) long, 25 Å (5 nm) across, and 35 Å (3.5 nm) high. Manufacturers C and D may be marketing solutions containing nothing larger than the hexamer but manufacturer A's product appears to contain polymers that are manyfold greater than the hexamer in size. Whether the observations reported here reflect differences in the manufactured prod-

uct or subsequent changes resulting from the shipping and storage of samples following manufacture is not known. Further studies are in progress to address these questions.

Manufacturer B produces an insulin that apparently tends to form microcrystals. This microcrystalline content precludes direct size comparison with the other insulins studied. Although the results presented are preliminary, it would be interesting to determine the exact procedures in insulin extraction and formulation that cause the diversity in final particle size.

Quantitative studies on particle size and concentration are in progress, and it is anticipated that techniques currently under development will enable us to present histograms of the distribution of particle size rather than mean values for the predominant species. It is interesting to note that in our experience with insulin of different manufacturers in various insulin delivery systems, no differences were seen in their tendency to occlude the system. All insulins formed plugs that lead to blocking of the system's tubing.

ACKNOWLEDGMENTS

This work was supported by a negotiated contract #NO1-AM-9-2201 from the National Institutes of Health, Bethesda, Maryland.

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