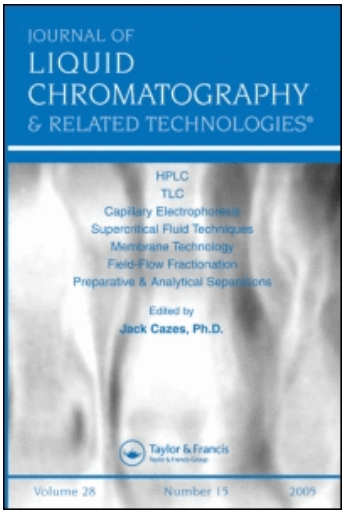

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HPLC Gel-Filtration of Insulin During Short and Long Time Infusion by Artificial Delivery Systems

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To cite this Article Schrader, E. and Pfeiffer, E. F.(1985) 'HPLC Gel-Filtration of Insulin During Short and Long Time Infusion by Artificial Delivery Systems', Journal of Liquid Chromatography & Related Technologies, 8: 6, 1121 — 1137
To link to this Article: DOI: 10.1080/01483918508067132
URL: http://dx.doi.org/10.1080/01483918508067132

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(6), 1121-1137 (1985)

HPLC GEL-FILTRATION OF INSULIN DURING SHORT AND LONG TIME INFUSION BY ARTIFICIAL DELIVERY SYSTEMS

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SUMMARY:

We examined with High Performance Liquid Chromatography (HPLC) gel-filtration the aggregational behaviour of insulin during application by artificial delivery systems.

The short time infusion was performed by an autosyringe pump and the long time infusion was performed by a Siemens pump. For comparison we collected samples of about 1 ml and determined the radioimmunological activity in order to verify the results of the HPLC gel-filtration.

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 0148-3919/85/0806-1121$3.50/0

The chromatograms, performed at 37°C and at ambient temperature, showed no aggregates of a higher molecular weight.

We observed sedimentation only once during long time infusion, however, the immediately performed gel-filtration surprisingly showed at no time an aggregate of a higher molecular weight, but the quantity of insulin was obviously reduced.

A different length of the catheter systems used did not promote the aggregational behaviour.

Furtheron, the absorbed quantity of insulin depends on the length of the catheter system used. The material of the catheters was polyethylene.

INTRODUCTION:

The tendency of insulin to aggregate in artificial delivery systems requires frequent replacement (8), or flushing of the insulin reservoir, or catheter systems. Insulin has an inherent tendency to polymerize and form large molecular weighty aggregates which do not equilibrate rapidly with the rest of the solution. Besides the danger of occlusion of the insulin infusion devices, it has been suggested (8) that the heterogeneous mixture does not allow delivery of the hormone within the fine limits of tolerance which are required to maintain euglycemia.

Therefore we examined with High Performance Liquid Chromatography (HPLC), using the method of gel-filtration chromatography, the formation of monomers, dimers and tetramers or

other insulin aggregates of higher molecular weight during the application by artificial delivery systems.
Furthermore, we examined the influence of the different lengths of catheter devices.
In addition we performed radioimmunological investigations in order to confirm the results of gel-filtration.

MATERIAL AND METHODS:

Apparatus:

The apparatus consisted of a programmer series 200 (Kontron, Munich, Germany), a switch valve LMV 870 (Kontron), two LC 414 pumps (Kontron), a high temperature oven (Knauer KG, Bad Homburg,Germany), an Uvikon photometer 720 LC (Kontron) and a Shimadzu integrator C-R1B (Kontron).

Insulins:

We examined neutral Velasulin^{R} Nordisk (Gentofte, Denmark) and soluble Hoechst CS^{R} insulin (pH 3.6)(Hoechst AG, Frankfurt, Germany).
We are grateful for the generous gift from Hoechst AG of insulin which was stored for three years at 37°C. This preparation contained aggregates of insulin (personal communication Dr.Grau, Hoechst). The concentration of these insulins was 40 E/ml.

Method:

For the HPLC gel-filtration we worked with a TSK 125 column of Bio-Rad (Munich, Germany). Our experiments were performed at 37°C, flow rate 0.75 ml/min, wavelength 215 nm, measure-

ments of control at 280 nm; the recovery was 99 %, photometrically determined.

For the gel-filtration we used phosphate buffers, pH 7.40 (+/- 0.05), with the addition of 0.1 M NaCl. To test the quality of the column we regularly separated a mixture of Bio-Rad containing five calibration proteins. To calibrate the column we separated a mixture composed of Thyreoglobulin, IGg, Ovalbumin, Myoglobin and Cyancobalamin (figure 1).

To identify the peak position of the insulin monomer we determined the retention of pure crystalline human insulin, delivered by Eli Lilly GmbH (Gießen, Germany).

To show the separation of insulin aggregates by our method we tested the insulin formulation stored for three years at 37°C (figure 4). All the chemicals we worked with were of HPLC grade. We used water which was distilled after ion-exchange procedure. Before injecting the samples and the buffer, all were filtered by a filter RC55 (0.45 µm) from Schleicher & Schüll (Dassel, Germany). To avoid unspecific absorption of the filters, we compared the chromatographic experiments of unfiltered and of filtered samples. The solvents used were degassed by helium.

For the short time infusion (three days regularly) we examined Velasulin^{R} (Nordisk) taken from an auto-syringe pump. At the beginning and at a short time before the end of an application period, we examined the insulin with gel-filtration chromatography. The insulin was taken from the top of the catheter system. In addition we tested the influence of the length of the catheter. At one time we used a catheter

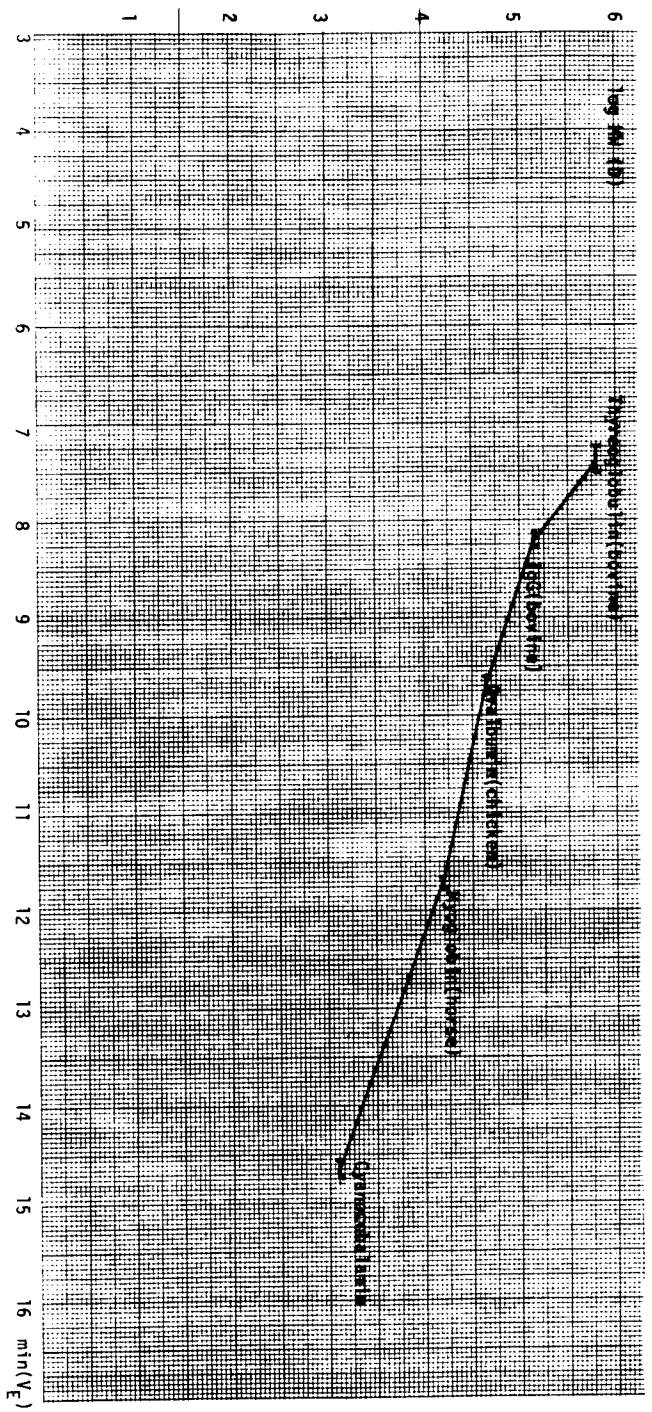

Fig.1: Calibration curve.TSK 125 column of Bio-Rad. Flow 0.75 ml/min, 215 nm, column temperature 37°C, phosphate buffer pH 7.40 and 0.1 M NaCl.

of 107 cm (cat.no.ASIV 27, material: polyethylene) and at the other time one of 60 cm.

For the long time infusion we examined CS insulin^{R} (Hoechst) delivered by a Siemens pump during four weeks. At the beginning and at the 9th, 14th and 25th day of the application period we examined a sample of insulin taken from the top of the catheter. The gel-filtration was performed with buffers pH 3.60 and 7.40. We used the buffer pH 3.60 because the CS insulin^{R} formulation is solved at pH 3.60.

To test the material of the catheter for its absorption of insulin, we filled an auto-syringe catheter (cat.no. ASIV 27) of 60 cm and another one of 107 cm with Velasulin^{R} which was mixed 1:5 with phosphate buffer pH 7.40. We rinsed out the contents of the tube into a sterile small bottle, then we analysed the sample by gel-filtration chromatography. The content of insulin before and after filling the catheter system was calculated by a Shimadzu integrator C-R1B. The tests were repeated several times.

All our tests were performed at the attenuation 2^{5} and 2^{3} mV/full scale of the Shimadzu integrator.

We performed the examinations during two independent application periods and we tested the two pumps with three patients to a pump. The insulin was injected into the skin of the abdomen. Each patient measured his own blood sugar level.

For the radioimmunological experiments we worked with an insulin kit from Sorin Biomedica (Saluggia, Italy).

Samples were collected manually at the outlet of the detec-

tor each minute; these samples were tested with the radio-immuno-assay. The lower limit of the calibration curve was 7 µE/ml.

RESULTS:

Before filling the insulin reservoirs of the two artificial delivery systems and at the end of the application periods, we examined the used insulin preparations with HPLC gel-filtration. During short time infusion with an auto-syringe pump, the delivered insulin (Velasulin^{R}) showed at no time a visible turbidness. The gel-filtration showed no aggregates of higher molecular weight (figure 2). The samples contained only insulin and the added phenol derivative.

During long time infusion, CS insulin^{R} (Hoechst) was delivered by a Siemens pump. During a change of the catheter system we obtained once a visible milky suspension remaining in the catheter. The immediately performed gel-filtration, pH 7.40, showed no aggregate of a higher molecular weight, but the quantity of insulin was obviously reduced (figure 3). At pH 3.60 we did not achieve a good result from separation.

We emphasize that our determinations of the molecular weight of insulin were measured between the limits of 5,000 and 7,000 Daltons.

When compared with other application periods, there were no differences in the blood sugar levels; even values up to 350 mg% were measured and at one time a blood sugar level of 40 mg% was noticed. A different length of the used polyethy-

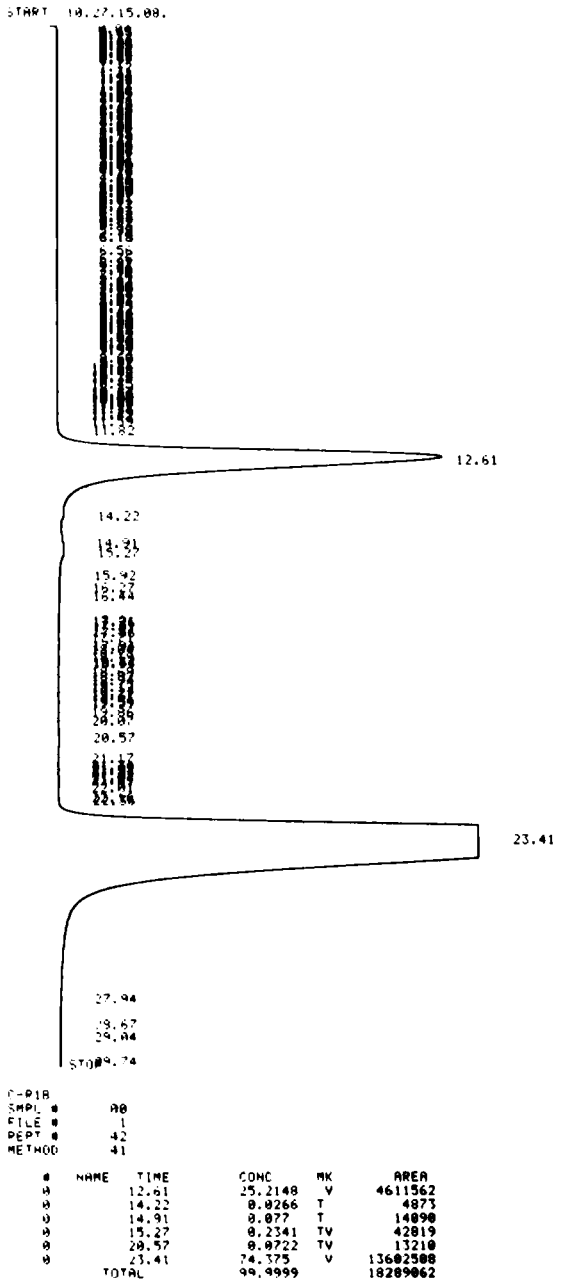

Fig.2. Typical chromatogram of gel-filtration. Column temperature 37°C, TSK 125 column of Bio-Rad. Flow 0.75 ml/min, phosphate buffer pH 7.40 and 0.1 M NaCl, 215 nm. Att 2^5 mV/full scale; 20 µl injection volume; at 12.61 min the insulin peak, at 23.41 min the phenol peak.

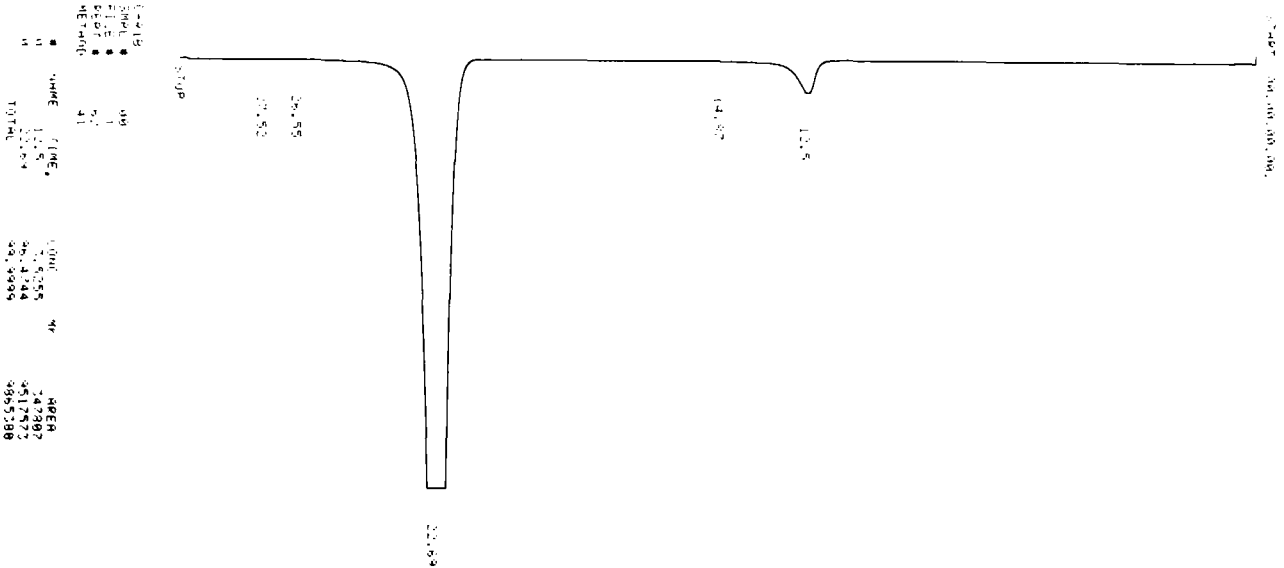

Fig.3: Gel-filtration of the visible milky suspension staying in the catheter. Column temperature 37°C. The insulin peak is reduced. TSK 125 column of Bio-Rad, 215 nm, Flow 0.75 ml/min, phosphate buffer pH 7.40 and 0.1 M NaCl. Att 2^5 mV/full scale; 20 µl injection volume, at 12.5 min the insulin peak.

lene catheter did not influence the self-aggregational behaviour.

Finally we attempted to find out how much insulin was absorbed by a catheter system with a length of 60 cm and another one with a length of 107 cm. Under the conditions mentioned above, it was shown that the catheter with a length of 60 cm absorbed 6.15 % and the catheter with a length of 107 cm absorbed 12.2 % of the insulin concentration. The deviation from test to test was 0.5 %. It can be concluded that the length of the catheter material used influences the quantity of the absorbed insulin.

We show with the insulin stored for three years at 37°C the possibility to separate aggregates of insulin (figure 4).

For the radioimmuno-assay, the sample collected at the retention time of the insulin showed full radioactivity which was widely outside the linear part of the calibration curve. The lower activity of the other samples was not readable on the linear part of the calibration curve. These facts supported our results of gel-filtration.

DISCUSSION:

The aggregation of insulin in artificial delivery systems has been well discussed during the last years (8). Insulin solutions seem to prefer aggregating in solution instead of forming complex coatings on the surfaces of pumping systems. To examine the precipitation, the pumps and all parts concerned were viewed with a scanning electron microscope (SEM) (8). The materials tested included polytetra-

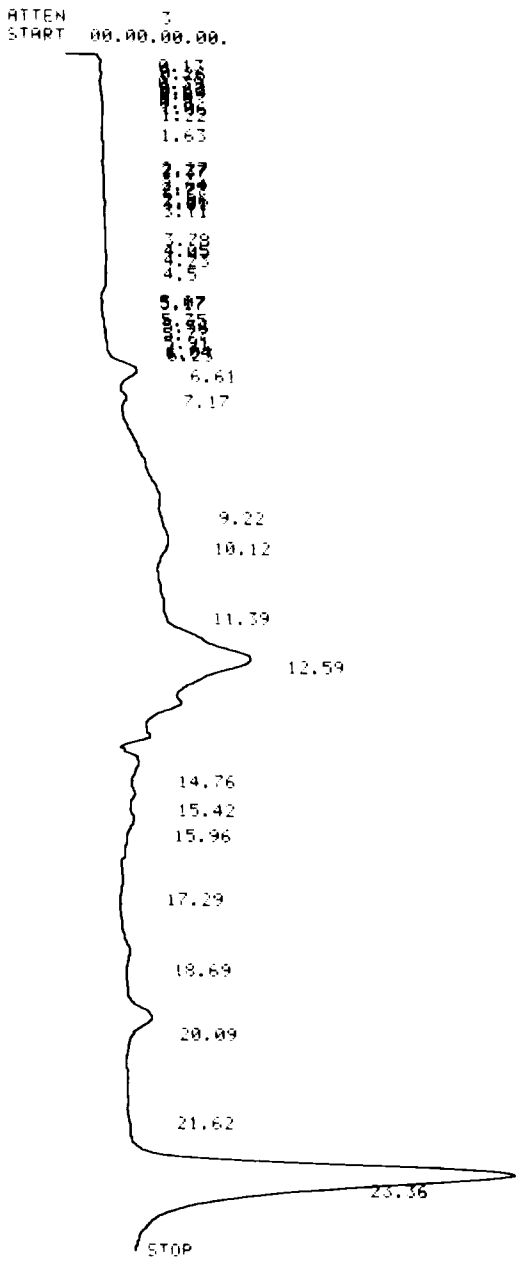

Fig.4: Gel-filtration of insulin which was stored during three years at 37°C. TSK 125 column of Bio-Rad, 215 nm, Flow 0.75 ml/min; phosphate buffer pH 7.40 and 0.1 M NaCl. The retention time of insulin at 12.59 is in good accordance with other figures. Att 2^3 mV/full scale.

fluoroethylene, titanium, silicone rubber, glass, polyvinylchloride and cellulose acetate. Substantial amounts of insulin were found 1-2 cm down stream from the pump, all other sections were free of deposition. Precipitated material produced by shaking neutral pork insulin was submitted to electrophoresis at pH 3.0. This analysis indicated the presence of very high molecular weight material, several high molecular weight insulin-derived materials, insulin itself and desamido insulins (8).

We investigated this problem with HPLC gel-filtration and to achieve a better control we used radioimmunological investigations as well. Due to the lack of a refractometer we could not establish the calibration curve with dextrans or polyethylene glycols. To determine the elution volume of substances with a low molecular weight we used cyanocobalamin because other tested substances such as bacitracin, actinomycin C were not pure.

Gel-filtration with HPLC is documented in the literature; Welinder (13) separated at ambient temperature bovine and porcine insulin (crystallized twice). To receive results with good reproduction, a constant temperature during the experiments is very important (5,9). Our experiments took this demand into consideration. The use of TSK 125 columns for molecular weight estimation is documented in the literature (11). Richter et al. determined with TSK 125 columns the molecular weight in the range from 1,000 to 10,000 with accuracy of +/- 15 % and up to 17.800 of +/- 20 %. In addition, we used the same peptides for calibration as they did.

With regard to the gel-filtration we did not find any aggregates of higher molecular weight of the insulin molecule. Under the retentions of the insulin we always detected one peak, even if we repeated the test at 280 nm. With gel-filtration the molecular weight of proteins cannot be determined exactly because the retentions of the proteins are influenced by pH, ionic strength, hydrophobic interactions and ion-exchanges between protein and stationary material. For example, the elutionary volume of catalase and ferritin is smaller than expected; this can be improved with a higher ionic strength of the buffer (3). These facts are responsible for the deviations of the determined molecular weights from the true molecular weight (3,5,9). The calibration curve is S-shaped because the gel becomes uniformly permeable to molecules which are too large or too small for the exclusion size. In order to make the curve well readable, we drew the log MW against the elution volume. We emphasize that the range of the molecular weight was within the limits of 5,000 and 7,000 Daltons.

The anomalous stability of insulin at very high pressure - contrary to other hormones - is favourable to our investigations (10). Fisher and Porter (4) discussed that purified crystalline bovine insulin degraded by two mechanisms: deamidation and polymerization.They determined for both mechanisms the activation energy. The polymerization of insulin during application with a pump can cause a loss of bioavailability or even an obstruction of internal pump parts. According to our results, we could not detect a measurable

polymerization during an application period of a few days. The literature (4) documents that after a storage of three months at 5°C the percentage of high molecular weight material is 0.19 % and at 37°C the percentage is 16.5 %. The investigated insulin was crystalline bovine insulin. The percentage of aggregates under continuous motion is not well documented in the literature.

Infrared dichroism and wide-angel X-ray diffraction clearly demonstrate the cross-ß-structure of the submicroscopic fibrils. These fibrils were formed by the polymerized globular protein insulin (2).

Due to the limited sensitivity of our UV-detector we could not conclude that all aggregated insulin is detected. Schlichtkrull et al.(12) divided crystalline insulin, fractionated by common gel-filtration chromatography, according to molecular weight into the high-molecular weight (mol.wt. – 15,000) a-component, the b-component and the c-component. The c-peak consists of arginine-insulins, insulin ethyl esters, the insulin and monodesamidoinsulin; the b-peak consits of proinsulin, intermediates and the dimer; the a-peak has not been identified. During the application periods we detected only the c-component, but we could not decide between deamidated insulin, insulin esters and insulin. The possible presence of these insulin derivates is confirmed by the literature (6). The radioimmuno-assay does not support this decision because the amino acid sequence is identical over a wide range. Our method of sample collection is not accurate enough; with an electronic peak controlled

fractions collector it would be possible to separate the peak further with electrophoresis.

We do not agree with Irsigler (7), who proposed that the precipitation of insulin in infusion devices seems to be an ununavoidable consequence of continuous motion. Also the examination of the visible sedimentation in the catheter system results in no aggregates of a higher molecular weight. This sedimentation is irritating the patients but only the quantity of insulin is reduced. During short time infusion (a few days) the plugging of delivery devices seems to be a low risk. In addition Blackshear et al. (1) described that insulin precipitated and occluded nine pumps, which were implanted in normal dogs, within 43 days. In contrast, pumps containing insulin mixed with 80 % glycerol functioned normally for more than 250 days. The biological potency of insulin is reduced during storage according to a first order reaction (12). Before the rate at which insulin loses its biological activity in delivery systems can be assessed, it is obvious that anti-aggregating diluents must be subjected to long-term testing both in vitro and in vivo.

Acknowledgment:

The diligent technical assistance provided by Mr H.Zier is gratefully acknowledged.

Supported by Foundation Herbert-Weishaupt e.V.

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