ELSEVIER

International Journal of Pharmaceutics 217 (2001) 71–78

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

A study of factors controlling dissolution kinetics of zinc complexed protein suspensions in various ionic species

Sunil Prabhu [a,*], Arthur I. Jacknowitz [b], Paula Jo Stout [b]

[a] *College of Pharmacy, Western University of Health Sciences, 309 E, 2nd Street, Pomona, CA 91766, USA*
[b] *School of Pharmacy, West Virginia University, Health Sciences Center, Morgantown, WV 26506, USA*

Received 17 August 2000; received in revised form 17 January 2001; accepted 18 January 2001

Abstract

The presence of bound and unbound zinc in the crystal matrix of protein suspensions helps physically stabilize the crystal and limits the dissolution of the drug. In case of zinc insulin suspensions, dissolution can be promoted by complexation of zinc with an ionic species for which the zinc has a greater affinity, these complexing ions having either formulation and/or physiological relevance. The purpose of this work was to use ligand-complexed formulations of insulin suspensions to gain an understanding of these products' dissolution performance and to establish a fundamental understanding of the rate-limiting steps in zinc insulin dissolution. Our group has suggested that the critical factors to zinc insulin dissolution are: (1) chemical complexation (zinc with ionic species); and (2) drug transport (insulin diffusion and solubility). Dissolution studies conducted using different ionic species (acetate, phosphate, citrate and EDTA) in a spin-filter device demonstrated a rank order correlation for different sources of zinc insulin; it was observed that human zinc insulin dissolved faster than bovine insulin, these differences attributed to their binding properties and the respective affinities to the various ionic species used. Also, as the amount of crystallinity increased in a formulation, a rank order increase in dissolution times was observed. The project also identified a sensitive and reproducible dissolution testing methodology. Overall, this study demonstrated that: (1) the complexation rate-limiting step was more significant in the dissolution of zinc insulin than the diffusion rate-limiting step; and (2) that dissolution kinetics depended primarily on the source and solid state differences and the binding affinities of the zinc insulin.

Keywords: Dissolution; Zinc–insulin suspensions; Ionic species; Complexation; Diffusion

1. Introduction

The presence of divalent metal ions serves a variety of functions in proteins, the most important of which is to enhance the stability of the protein in the conformation required for biological function (Christianson, 1991). The addition of appropriate amounts of zinc ions to protein preparations, such as recombinant insulin, has been shown to also achieve a higher degree of purification and control over the duration of ac-

* Corresponding author. Tel.: +1-909-4695550; fax: +1-909-4695539.
E-mail address: sprabhu@westernu.edu (S. Prabhu).

0378-5173/01/$ - see front matter
PII: S0378-5173(01)00600-7

tion of the insulin (Schlichtkrull, 1975). With regard to insulin and metal ion forming complexes, early studies recognized the importance of zinc for the crystallization of insulin in the rhombohedral form (Hallas-Moller, 1956). It was shown that insulin preparations with protracted effect could be obtained by addition of small amounts of zinc ions. Furthermore, it was demonstrated that the degree of protraction at the same zinc concentration depended on the physical state of the suspended insulin particles, amorphous insulin particles having a shorter time of action than crystalline insulin particles (Schlichtkrull, 1961). While the behavior of zinc insulin preparations post crystallization has been well characterized (Brange, 1987), the process by which these solids dissolve is not as clearly understood. The understanding of the mechanism by which dissolution controls the release of the drug has the potential utility of identifying those formulation and/or process variables which may promote zinc insulin solution and in developing appropriate and sensitive testing methodologies.

Dissolution may, in fact, be one of the rate-limiting factors for the absorption of subcutaneously injected zinc insulin suspensions (Hildebrandt et al., 1985). In this regard, an understanding of the mechanisms by which these ligand-complexed protein suspensions dissolve, and the factors which influence the kinetics of this process is of interest. Initial studies conducted in our laboratory showed that complexation of zinc with an ionic species to which zinc has greater affinity than for insulin promotes dissolution via both, a zinc concentration gradient effect and loss of crystal stability upon loss of the zinc ion (Prabhu and Stout, 1991; Prabhu, 1996). Hence, the overall objective of the current study was to assess the in vitro dissolution of ligand-complexed protein suspension formulations in various ionic species, to establish a fundamental understanding of the rate-limiting steps in zinc insulin dissolution. As such, steps which may be controlling the release kinetics of zinc insulin are: (1) chemical complexation, whereby the zinc ion complexes with an ionic species to which it has a greater affinity and; (2) insulin diffusion, in which the insulin molecule transports from the solid surface into solution. Thus, studies were conducted to characterize the interrelationship of each of the above steps, assess the role of zinc in this process and elucidate differences in release kinetics of various zinc insulin sources. Additionally, the current studies determined the sensitivity and reproducibility of the chosen dissolution method.

2. Theoretical

The appropriateness of an elementary transport model for describing zinc–insulin dissolution can be tested by using the following assumptions applied to the model: (1) zinc ions complex with buffer ions for which they have a greater affinity than for insulin (Fig. 1), leading to the removal of the metal ion, rendering the protein more soluble and; (2) diffusion of the protein from the surface of the solid into solution (Fig. 2). Mathematically, a quasi-steady state model (Cussler, 1986) was used to represent the decrease in mass of the dissolving crystals. Here, the rate of loss of mass is equal to the steady-state flux,

$$\mathrm{d}M/\mathrm{d}t = -AJ \tag{1}$$

where, $\mathrm{d}M/\mathrm{d}t$ is the dissolution rate, A is the surface area and J is the flux. The steady state flux, J, maybe represented as,

$$J = \mathrm{Dc}/(R_r + R_d) \tag{2}$$

where, flux (J) is equal to the driving force for dissolution (Dc for drug particle) divided by the total resistance ($R_r + R_d$). Assuming linear resis-

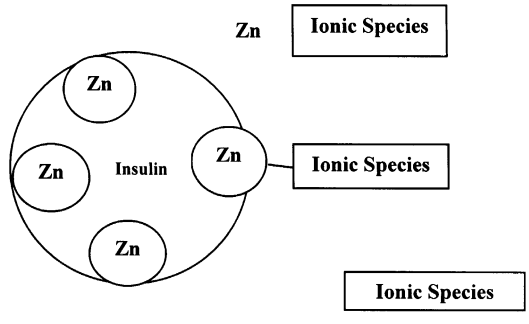

Fig. 1. Schematic representation of the complexation step in the dissolution of zinc–insulin suspensions.

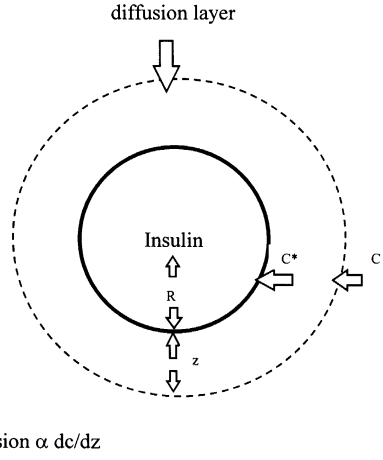

Fig. 2. Schematic representation of the diffusion step in the dissolution of zinc–insulin suspensions.

tance, the denominator term is the sum of the individual resistances in series. The driving force (Dc) for the release of drug is the concentration difference of insulin between the surface of the drug particle and the bulk solution. It is assumed that the concentration at the interface between two phases are at equilibrium and that the concentration at the solid surface is the solute equilibrium solubility. It is common to refer to the reciprocal of the total resistance as overall mass transfer coefficient, K, which is defined here as,

$$1/K = R_r + R_d = 1/k_r + 1/k_d \quad (3)$$

where, k_r and k_d are constants for the reaction and diffusion step, respectively. The reciprocal of constants, $1/k_r$ and $1/k_{d,}$ represent the reaction and diffusion resistances, respectively. The above relationship was used to assess the influence of either resistance term on the dissolution of zinc complexed insulin suspensions.

Within the framework of the above transport model, the following hypotheses was tested: (1) if the complexation step is critical in zinc insulin dissolution, then release kinetics should proceed at a rate: (i) which correlates with the zinc's affinity for the ionic species present in the dissolution medium; and (ii) which correlates with zinc's degree of association with the insulin molecule; and (2) if the diffusion step is critical to the dissolution of zinc insulin, then release kinetics should proceed at the same rate under controlled flow conditions of the device, regardless of the zinc ion's affinity towards the insulin molecule. Thus, these critical steps in zinc insulin dissolution were determined based on the proposed elementary transport model.

3. Materials and methods

3.1. Materials

Zinc complexed recombinant human and bovine insulin suspensions (amorphous, amorphous:crystalline mix and crystalline) were a gift from Eli Lilly and Company, (Indianapolis, IN). Salts of citric acid, acetate, phosphate and sodium hydroxide were purchased from Fisher Scientific (Fairlawn, NJ). Chelating agent ethylenediamine tetraacetic acid (EDTA) was purchased from Aldrich Chemicals (Milwaukee, WI).

3.2. Methods

3.2.1. Selection of ionic species

Ionic species were selected based on their increasing order of affinity toward the zinc metal ion (Table 1). Previous studies have shown varying affinities of the selected ionic species towards zinc metal ions; acetate buffer had the lowest affinity, with the affinities of phosphate and citrate and EDTA all increasing significantly in order of magnitude (Perrin and Dempsey, 1974; Ringbom, 1979). EDTA had an 18-fold increase in affinity towards zinc ions when compared to acetate buffer. The molar concentration of each ionic species was maintained at 0.026 M. This

Table 1
List of ionic species based on their increasing order of affinity towards the zinc metal ion

Ionic species	Association constants (M^{-1})
Acetate	3.3×10^{-2}
Phosphate	2.5×10^{2}
Citrate	6.9×10^{4}
EDTA	1.0×10^{16}

concentration was determined to be adequate for sensitivity of the dissolution runs since it is approximately two orders of magnitude greater than the total concentration of zinc ions (bound and unbound) present under the experimental conditions (the molar concentration of zinc ion: 1.2×10^{-4} M; insulin: 3.3×10^{-5} M). Therefore, theoretically the zinc ions were 'swamped' by the ionic species present for complexation.

3.2.2. Characteristics of zinc insulin suspension formulations

Zinc insulin suspensions were obtained from different sources (human and bovine); three different types of formulations namely, semilente (amorphous, short acting), lente (amorphous/crystalline (3:7) mix, intermediate acting) and ultralente (crystalline, long acting) suspensions were used. The characteristics of insulin formulations included four zinc insulin, whereby four zinc ions were bound per insulin hexamer unit. Each of these zinc ions was coordinated with two imidazole groups within the insulin molecule. Of the four-zinc ions, two were more tightly bound such that there existed both, a high [10^{13} (bovine), 10^{6} (human)] and low binding [10^{8} (bovine), 10^{4} (human)] affinity of zinc within each insulin hexamer (Brange and Langkjaer, 1993). Each 10 ml sample consisted of 100 units/ml (corresponding to approximately 3.8 mg/ml) of insulin. The size of the crystals was carefully controlled at 25 μ whereas a rhombohedral shape was retained for all zinc insulin crystals. All samples were formulated free of preservatives.

3.2.3. Dissolution studies

All dissolution studies were conducted using the spin-filter dissolution apparatus which has previously been shown to be appropriate for testing suspension formulations (Shah et al., 1973). The temperature and pH of the dissolution medium was maintained at 25 ± 0.5°C and 7.4, respectively. A measured amount of sample (10 ml, 100 U/ml) was introduced into the dissolution flask containing 180 ml of the dissolution medium. Prior to injection of sample, the dissolution apparatus was set up to assay samples on a continuous basis. A peristaltic pump was connected to a flow-through UV spectrophotometric cell placed inside an UV–VIS spectrophotometer (DU-65, Beckman Instruments, Philadelphia, PA). The pump rate was maintained at 15 ml/min while speed of stirring was set at 300 rpm. At predetermined intervals, samples were analyzed in the UV–VIS spectrophotometer at 280 nm for the release of insulin. All studies were performed in triplicate. Using the procedure outlined above, the following variables for zinc insulin were tested: (1) effect of different ionic species; (2) effect of diffusion as influenced by increasing stirring speeds; (3) solid state differences; and (4) source differences.

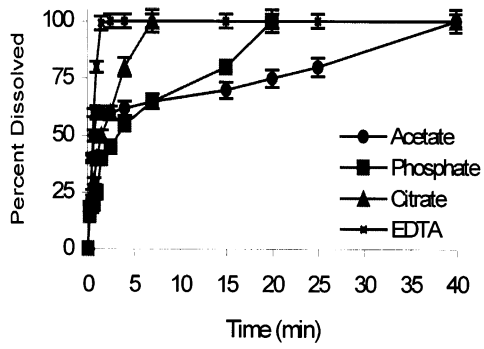

Fig. 3. Dissolution profiles of human zinc–insulin suspensions in different ionic species (pH 7.4, 25°C ± 0.5).

4. Results and discussion

The dissolution curves for human and bovine zinc insulin in various ionic species is shown in Figs. 3 and 4. As noted from the graphs, a rank order correlation was observed indicating the affinity that each ionic species had for zinc, regardless of the source of insulin. Both profiles illustrated that the ionic species with greater affinity towards zinc provided faster release kinetics. From Fig. 3, it was observed that approximately 50% of the human zinc insulin drug were dissolved within the first few minutes of the dissolution run. The same phenomenon was also observed in case of bovine insulin dissolution (Fig. 4). The probable cause for this occurrence was most likely a dilution effect by the dissolution medium, resulting in the loss of the free zinc with

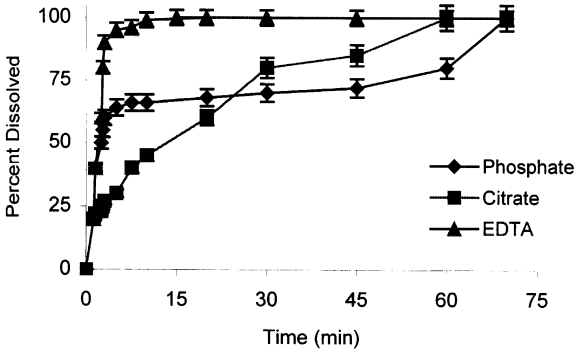

Fig. 4. Dissolution profiles of bovine zinc–insulin suspensions in different ionic species (pH 7.4, 25°C ± 0.5).

dilution and the generation of a zinc concentration gradient in the dissolution medium. However, the initial dissolution rates for human and bovine zinc insulin were observed to be different. As stated earlier, the presence of two different degrees of binding affinities (low and high) of zinc ions within each species of insulin may be contributing to the difference in initial dissolution rates. Since, the low binding affinity constant of bovine insulin is greater than that of human insulin, this causes the initial dissolution of bovine insulin to slow down considerably when compared to human insulin. The remaining 50% of the protein drug subsequently dissolves as per the higher zinc binding affinities for each species and the increasing attraction of the selected ionic species towards the zinc ions.

Based on the proposed mathematical model, the dissolution profiles showed that zinc insulins demonstrate different release kinetics in the presence of various ionic species. In case of human crystalline zinc insulin, the release kinetics were seen to be fastest in EDTA causing human crystalline zinc insulin to completely dissolve in 2 min, and slowest in acetate based buffers in which the insulin did not go into solution until the end of 40 min. Similarly, for bovine crystalline zinc insulin, EDTA demonstrated the fastest kinetics ($T_{100\%}$: 4.5 min) whereas phosphate demonstrated the slowest release of zinc insulin ($T_{100\%}$: $\sim$70 min) (Table 2). Acetate dissolution data were not found to be reproducible with bovine zinc insulin as the kinetics were extremely slow therefore, this data was not reported. The slower release kinetics of the bovine zinc insulin molecule was attributed to a stronger binding affinity to the zinc metal ion (affinity: 10^{13}/M) when compared to human zinc insulin (affinity: 10^{6}/M). These results suggested that the reaction resistance term (i.e. complexation) was significant to the process of zinc insulin dissolution. Further analysis was performed in order to correlate the dissolution rate of human zinc insulin with complexation by plotting the calculated dissolution rates against the zinc association constant values (Fig. 5), assuming a constant surface area of all samples tested. This analysis showed that the relationship between dissolution and ion affinity was not linear, especially for the citrate ion. Therefore, release kinetics of the zinc insulins were not predictable based solely on complexation affinity. Although the significance of the complexation step was further confirmed from these studies, the analysis also suggested that complexation may not be the only controlling factor in zinc insulin dissolution.

Table 2
Comparative data of percent dissolved human and bovine zinc insulin, indifferent ionic species as a function of time

Ionic Species	Percent dissolved			
	$T_{50\%}$ (min)		$T_{100\%}$ (min)	
	Human Zn insulin	Bovine Zn insulin	Human Zn insulin	Bovine Zn insulin
Acetate	1.5	N/A	40	N/A
Phosphate	2.5	3.0	20	72
Citrate	1.5	15.0	7.5	63
EDTA	0.25	2.5	2.0	4.5

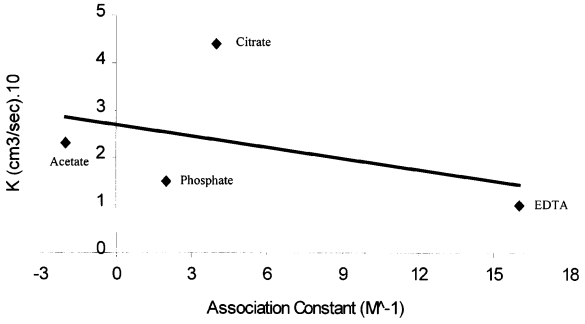

Fig. 5. Relationship of dissolution rate constants to complexation association constants: human zinc insulin.

From the results obtained above, the citrate buffer species was identified as the ideal dissolution medium for subsequent experiments. This was due to the fact that EDTA demonstrated rapid kinetics and hence its role as a dissolution medium was questionable due to limits in sensitivity. Acetate and phosphate buffers had extremely slow dissolution kinetics and therefore were not feasible as a choice for quality control and data reproducibility. The citrate-buffered medium demonstrated analytical sensitivity to the zinc ion and upon complexation provided a reasonable time frame of release of the insulin, both in case of human and bovine zinc insulins, dissolving completely within 7.5 and ~ 60 min (Table 2) of the dissolution runs, respectively. The citrate medium also showed adequate influence over the ligand metal ion zinc, to allow insulin to go into solution.

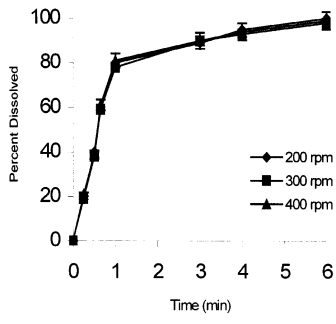

Fig. 6. Dissolution profiles of human zinc insulin as a function of increasing speeds of stirring (200–400 rpm).

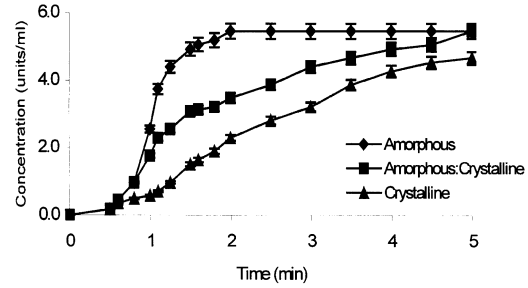

Fig. 7. Dissolution profiles of human zinc insulin in 0.026 M citrate buffer as a function of variable solid states.

Fig. 6 shows the potential influence of the diffusion resistance term in the dissolution of human zinc insulin. As noted in the hypothesis, if the mass transfer term (diffusional resistance) was significant, then increasing the speed of stirring would increase the rate of dissolution as a result of increased convective and shear effects on the crystals. From the graph, it was evident that increased stirring speeds had minimal effect on changing dissolution rates which suggested that it was probably the surface reaction step (i.e. complexation step) which controlled zinc insulin dissolution. Further studies were warranted to adequately determine the influence of the diffusion step.

The dissolution curves for various solid state formulations of human and bovine zinc insulin in citrate buffer species is shown in Figs. 7 and 8. As seen in Fig. 7, the dissolution test was initiated with the amorphous form of the human zinc

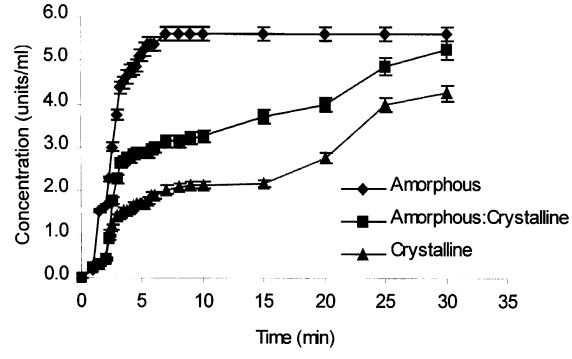

Fig. 8. Dissolution profiles of bovine zinc insulins in 0.026 M citrate buffer as a function of variable solid states.

insulin (semilente) which was injected into the dissolution medium. Subsequent runs with the crystalline (ultralente) and crystalline/amorphous mixture (lente) forms of human zinc insulins showed that while the amorphous form of the insulin dissolved in the fastest time, the kinetics of release were considerably slower as the crystalline content increased. A rank order correlation was observed whereby an increase in dissolution times was seen as content of crystals within each insulin formulation increased. Similar results were observed in bovine zinc insulin dissolution based on solid state differences as shown in Fig. 8. As expected, bovine zinc insulin dissolved at a slower rate than human insulin for reasons mentioned previously. However, similar to human insulin, the amorphous form of the bovine insulin went into solution faster than the crystalline or the mix of amorphous and crystalline insulin.

These results demonstrated that the assay method using the spin-filter device was capable of discriminating between formulations containing different amount of crystalline material. The sensitivity of this dissolution method had also been confirmed in a previous report (Stout et al., 1988) where a 'linear combination model' was applied to varying mixtures of amorphous to crystalline zinc insulin to describe their release kinetics. The dissolution rate of the mixtures was shown to be proportional to the relative amounts of amorphous/crystalline zinc insulin, with the initial rate more sensitive to the presence of the amorphous insulin and the terminal rate to the crystalline insulin, respectively.

Fig. 9 shows the percent dissolved-time curve comparison between two different sources of insulins, human and bovine. Since the bovine insulin molecule had a higher affinity toward the zinc metal ion than the human insulin molecule [10^{13} (Bovine), 10^{6} (Human)], it was expected that bovine zinc insulin would demonstrate slower release kinetics than human zinc insulin. This was confirmed when bovine insulin showed significantly slower dissolution ($T_{100\%}$: 60 min) than human insulin ($T_{100\%}$: < 10 min). The differences in dissolution rate between insulin of

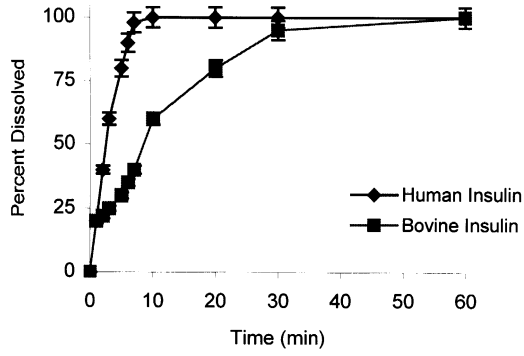

Fig. 9. Dissolution profiles of zinc insulins as a function of variable source.

varying sources also support the notion that zinc may exhibit different types of binding to insulins of different amino acid chain sequences, and are consistent with the in vivo performance of the two products.

In conclusion, these studies determined that the dissolution of zinc insulin is a function of two rate-limiting steps, complexation and diffusion, in which the former step was more dominating than the latter, in the overall dissolution process. The relative magnitude of influence of each of the rate-limiting steps to zinc insulin dissolution was (Prabhu, 1996) calculated to show that the complexation rate limiting step has a three-fold higher influence on the dissolution process than the diffusion rate limiting step. In terms of device sensitivity and method reproducibility, these studies demonstrated that the methodology was very sensitive to changes in source and structure of the zinc insulin formulations making it an ideal testing methodology for metal ion complexed protein formulations.

Acknowledgements

The authors acknowledge with gratitude the United States Pharmacopeial Convention Inc., Rockville, MD for awarding two successive fellowships to Sunil Prabhu in support of completion of this project.

References

Brange, J., 1987. Galenics of Insulin: The Physicochemical and Pharmaceutical Aspects of Insulin and Insulin Preparations, 1st. Springer-Verlag, Berlin, pp. 28–64.

Brange, J., Langkjaer, L., 1993. Insulin structure and stability. In: Wang, Y.J., Pearlman, R. (Eds.), Stability and Characterization of Protein and Peptide Drugs. Plenum Press, New York, pp. 315–350.

Christianson, D., 1991. Structural biology of zinc. Adv. Protein Chem 42, 281–355.

Cussler, E.L., 1986. Diffusion: Mass Transfer–Fluid Systems. Cambridge University Press, New York.

Hallas-Moller, K., 1956. Crystalline and amorphous insulin-zinc compounds with prolonged action. Diabetes 5, 7–17.

Hildebrandt, P., Sejrsen, P., Nielsen, S.L., Birch, K., Sestoft, L., 1985. Diffusion and polymerization determines the insulin absorption from subcutaneous tissue in diabetic patients. Scand. J. Clin. Lab. Invest. 45, 685–690.

Perrin, D.D., Dempsey, D., 1974. Buffers for pH and metal ion control. Chapman and Hall, NY.

Prabhu, S., Stout, P.J., 1991. Influence of insulin source and buffer species on crystal zinc insulin dissolution kinetics. Pharm. Res. 8 (10), 84.

Prabhu, S., 1996. Development and Assessment of a Standard Dissolution Methodology for Protein-Based Injectable Suspensions. Dissertation, West Virginia University, UMI Dissertation Services, Ann Arbor, Michigan 48106.

Ringbom, A., 1979. Complexation in Analytical Chemistry, vol. 2. Krieger Publishing Co., NY, pp. 215–224.

Schlichtkrull, J., 1975. Insulin preparations with prolonged effect. Handbook Exp. Pharmacol. 1, 115–132.

Schlichtkrull, J., 1961. Insulin crystals: chemical and biological studies on insulin crystals and insulin zinc suspensions. Acta Chem. Scand. 10, 1459.

Shah, A.C., Peot, C.B., Fuchs, J., 1973. Design and evaluation of a rotating filter-stationary basket in vitro dissolution test apparatus. I. Fixed fluid volume system. J. Pharm. Sci. 62 (4), 671.

Stout, P.J., Mauger, K., Burns, K., Mauger J.W., 1988. Dissolution characteristics of changing mixtures of amorphous:crystalline humulin zinc insulin, Technical report to Eli Lilly and Co., Indianapolis, IN.