Precipitation of Insulinotropin in the Presence of Protamine: Effect of Phenol and Zinc on the Isophane Ratio and the Insulinotropin Concentration in the Supernatant

Yesook Kim[1,2] and Carol A. Rose[1]

Received January 30, 1995; accepted March 10, 1995

Purpose. The purpose of this study is to determine the minimum quantity of protamine required for the completion of insulinotropin precipitation under different precipitation conditions. ***Methods.*** The experiments involved combining insulinotropin with varying concentrations of protamine in pH 7.2 phosphate buffered saline and analyzing the concentrations of both proteins in the supernatant. Isophane ratio (the protamine/insulinotropin molar ratio that results in a minimum total protein concentration in the supernatant) was determined for different precipitation conditions. ***Results.*** When neutral solutions of insulinotropin (p*I* 5.3) and protamine (p*I* 13.8) were combined, precipitation did not occur. However, in the presence of phenol and/or zinc, insulinotropin co-precipitated with protamine. In the presence of phenol, the isophane ratio and the insulinotropin concentration in the supernatant were determined to be 0.08 and 0.18 mg/ml, respectively. In the presence of zinc, the isophane ratio increased with zinc concentration, apparently from the precipitation of protamine in the presence of zinc. The isophane ratio and the insulinotropin concentration in the supernatant were 0.13 and 0.13 mg/ml, respectively, when the zinc/insulinotropin molar ratio was one. In the presence of phenol and zinc with the zinc/insulinotropin molar ratio of 1.0, the isophane ratio and the insulinotropin concentration in the supernatant were 0.11 and 1 μg/ml, respectively. ***Conclusions.*** A method to determine the isophane ratio of protamine/insulinotropin precipitation was developed to determine the minimum quantity of protamine required for the completion of insulinotropin precipitation under different precipitation conditions. A synergistic effect between phenol and zinc on the precipitation of insulinotropin in the presence of protamine was found.

KEY WORDS: insulinotropin (glucagon-like peptide I-(7-37)); protamine; phenol; zinc; isophane ratio.

INTRODUCTION

Protamine is the generic name of a group of highly basic proteins (1). Commercially available protamines are isolated from fish sperm, and usually are obtained as the sulfate salt. Protamines are used for heparin neutralization in cardiac procedures (2) and to treat heparin overdose (3). Another important medical application of protamine is its use as a complexing agent in the formulation of long-acting protein products such as Neutral Protamine Hagedorn (NPH) insulin (also called isophane insulin) (4), protamine zinc somatostatin (5–7), and protamine zinc glucagon (8,9). The addition of protamine induces the formation of sparingly soluble precipitates of the proteins, consequently the absorption of the proteins at the injection site is delayed. The dissolution of the proteins before absorption is presumably due to degradation of the protamine by the fibrinolytic tissue enzymes (10–13). Allergic reactions due to the protamine content of NPH preparations have, however, been reported (14,15), and it was concluded that protamine can be immunogenic in man and its use for medical purposes may lead to formation of antibodies (16–19). As a consequence, it is important that the minimum amount of protamine should be used in dosage forms.

When mixtures of a basic protein and an acidic protein are prepared with varying ratios of the two proteins, there will be a point, termed the "isophane ratio," at which the mutual precipitation is most nearly complete. Since the electric charge of the dissolved proteins depends upon the properties of the solution, stoichiometric precipitation cannot be expected. The ratio changes under given conditions such as pH and concentration of electrolytes. In 1938, Hagedorn developed a method to determine isophane ratios for insulin and protamine precipitation (20). This method is based on a nephelometric determination, and is currently used as an in-process control method for protamine insulin pharmaceutical products (21).

Simkin *et al.* (22) developed a spectrophotometric method to determine isophane ratio of lysozyme/insulin precipitation. They found that there was a significant discrepancy between the isophane ratio determined spectrophotometrically and the isophane ratio determined by the nephelometric method. Though the spectroscopic method is thought to be more reliable than the nephelometric method, it cannot be used for protamine/protein complexes, because protamine lacks aromatic residues. Use of high performance liquid chromatography (HPLC) for the isophane ratio determination has not been reported, although one would think that an HPLC method has advantages over the nephelometric method. The former requires substantially less proteins than the latter. Also, the HPLC method allows the proteins to be monitored separately by varying experimental conditions, so that the chemistry involved in the precipitation can be better understood.

Insulinotropin (glucagon-like peptide I-(7-37)) is a peptide containing 31 amino acid residues. It stimulates the secretion of the hormone insulin, and could be an important tool for the treatment of Type II diabetes mellitus (23–26). In an attempt to develop protracted insulinotropin preparations, we have recently studied the co-precipitation of insulinotropin with protamine in the presence of other excipients such as zinc or phenol. The pharmacokinetics of those formulations are reported elsewhere (27,28). As a part of the study, we developed a method to determine the isophane ratio for the protamine/insulinotropin precipitation by using HPLC. Here, the isophane ratio is the protamine/insulinotropin (Prt/Ins) molar ratio that results in a minimum total protein concentration in the supernatant. It is expected to change with excipients and the pH. A reproducible and accurate measurement of the isophane ratio is important because insufficient or excess protamine could affect the physical and chemical stability (29), and the pharmacokinetics (30) of a product.

[1] Pharmaceutical Research and Development, Central Research Division, Pfizer Inc., Groton, CT 06340.
[2] To whom correspondence should be addressed.

0724-8741/95/0900-000$07.50/0

MATERIALS AND METHODS

Materials

The insulinotropin (molecular weight 3,338, Pfizer lot 501713) used throughout this study was synthesized by Bachem California under contract to Pfizer. The protamine used was chum protamine sulfate (USP grade, Yuki Gosei lot F2101) received from Yuki Gosei Kogyo Co., Ltd., Tokyo, Japan. The molecular weights of the four chum protamines were determined by Hoffmann *et al.* (1). The average molecular weight (MW 4,217) was used for this study. Phenol (fused, USP grade) and zinc acetate dihydrate (USP grade) were obtained from J. T. Baker and Spectrum Chemicals, respectively. Deionized water which was further purified by a Milli-Q water system (Millipore Corp., Bedford, MA) was used. All other materials were of reagent grade.

Analytical HPLC for Insulinotropin

An analytical HPLC assay of insulinotropin was conducted with a Vydac Protein C4 column (Rainin Cat 214TP54), and the following gradient program, where *A* represents 0.1% trifluoroacetic acid (TFA) in water and *B* represents 0.1% TFA in acetonitrile.

Gradient Program:

Time (min)	0	5	30	35	37	46
% *A*	75	75	50	50	75	75
% *B*	25	25	50	50	25	25

The flow rate was set at 1 ml/min. The column was kept at ambient temperature (25°C). Detection was by UV at 215 nm. Insulinotropin retention time was approximately 23 min. Protamine did not interfere with the assay. The equipment used for the HPLC assay consisted of an LDC Consta-Metric 4100 solvent delivery system, a Bio-Rad Model AS-100 HRLC automatic sampling system, an LDC SpectroMonitor 4100 programmable variable wavelength detector, and a Spectra-Physics Chrom Jet integrator.

Analytical HPLC for Protamine

A reversed-phase HPLC for protamine was conducted with a 4.6 × 250 mm Zorbax C-8 column having a 150 Å pore size. The mobile phase was 2% acetonitrile and 98% of a 0.1 M monobasic sodium phosphate buffer adjusted with phosphoric acid to pH 2 (1). Elution was isocratic at 1 ml/min, and detection was by UV at 215 nm. The HPLC equipment used was the same as that used for the insulinotropin assay. The four individual chum protamines were separated by the HPLC method, and their retention times were approximately 6, 8, 10, and 18 min. For the assay, the total area of the four peaks was used. Insulinotropin was eluted close to the solvent peaks and did not interfere with the assay.

Precipitation

Stock solutions of insulinotropin (4 mg/ml), protamine (50.6 mg/ml), and phenol (40 mg/ml) were prepared in a 40 mM phosphate buffer (pH 7.2) containing 140 mM NaCl (PBS). A zinc stock solution (zinc 0.19 mg/ml) was made with water. All the stock solutions were filtered through 0.22 micron Millipore Millex-GV filters before use. The insulinotropin stock solution was used within 6 h after preparation, because self-association of the peptide can cause precipitation with time.

Insulinotropin suspensions were prepared in the presence of protamine with the aid of other precipitants such as phenol and/or zinc. In these experiments, the insulinotropin concentration was kept constant while protamine concentration was varied. A small portion of insulinotropin stock solution (375 μl) was placed into centrifuge tubes. The exact volumes of protamine, zinc, and/or phenol stock solutions to be added were calculated depending on the mixing ratios to be tested. Sufficient amount of PBS to produce a final volume of 1.5 ml was added to the tubes. Then, the calculated amount of protamine, zinc, and/or phenol stock solutions were added into the tubes almost simultaneously. These tubes were vortexed to mix the contents, and were allowed to sit at ambient temperature for 2 h. The samples were then centrifuged at 14,000 rpm for 10 min to separate the solids that had formed. The supernatants were filtered through a 0.22 micron Millipore Millex-GV filter and assayed for insulinotropin and protamine concentrations by HPLC. When necessary, samples were diluted with 0.01 N HCl for the protamine assay and with a 3:1 mixture of 0.1% TFA in water and 0.1% TFA in acetonitrile.

Circular Dichroism (CD) Measurements

CD spectra were obtained with a J-720 Jasco Circular Dichroic Spectropolarimeter in 0.01 cm path-length cells at 25°C.

Gel Electrophoretic Analysis

Insulinotopin was analyzed by isoelectric focusing on a Pharmacia-LKB Phast System using pH 3–9 Native Phast Gels.

RESULTS AND DISCUSSION

The chum salmon protamine is a mixture of four almost identical polypeptides, whose isoelectric points (p*I* 13.8) are unusually high (1). The p*I* of insulinotropin determined by isoelectric focusing is 5.3. When an insulinotropin solution in PBS was combined with a protamine solution in PBS, the resulting mixture remained clear at a protamine/insulinotropin (Prt/Ins) molar ratio from 0.1 to 2.2. To examine whether the two proteins interact with each other in solution, CD measurements were performed for three different samples, a 0.04 mM protamine solution in PBS, a 0.3 mM insulinotropin solution in PBS, and a solution containing both 0.3 mM insulinotropin and 0.04 mM protamine in PBS. They are shown in Figure 1. Insulinotropin shows a high content of α-helix, and protamine has hardly any secondary structure. A major change in the secondary structure of insulinotropin was observed in the presence of protamine. Insulinotropin and protamine appear to interact with each other, but whether they form a complex in solution under these conditions is not clear at the present time. Experiments are being conducted to gain a better understanding of the interaction between the two proteins in solution using

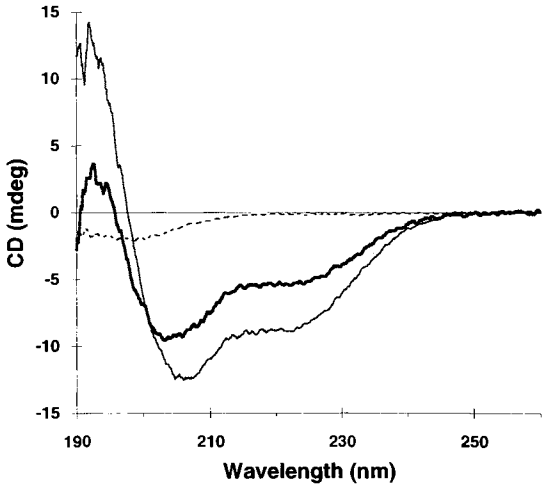

Fig. 1. Far UV CD spectra of protamine (---, 0.04 mM), insulinotropin (—, 0.3 mM), and insulinotropin (—, 0.3 mM) containing 0.04 mM protamine. The buffer used was pH 7.2 phosphate buffered saline. The path length of the cell used was 0.01 cm.

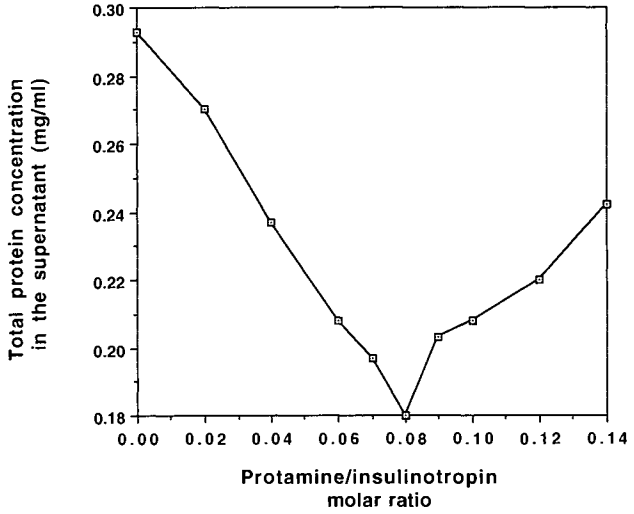

Fig. 3. The effect of protamine/insulinotropin molar ratio on the total protein concentration in the supernatant in the presence of 2.2 mg/ml phenol.

equilibrium analytical ultracentrifugation and microcalorimetry.

However, when the two protein solutions were combined in the presence of phenol (2.2 mg/ml), as shown in Figure 2, the concentration of insulinotropin in the supernatant decreased as the Prt/Ins ratio increased. Initially, the insulinotropin concentration dropped sharply with increasing Prt/Ins ratio. When the Prt/Ins ratio exceeded 0.09, the rate of decrease was diminished. On the other hand, protamine was not detectable in the solution until the Prt/Ins ratio reached 0.09, but it sharply increased with further increases in the Prt/Ins ratio. The sum of the two protein concentrations in the supernatant is plotted in Figure 3. A sharp *v*-shaped curve was obtained. The total protein concentration was lowest at the Prt/Ins molar ratio of 0.08. Thus, the isophane ratio of protamine/insulinotropin was determined to be 0.08 in the presence of 2.2 mg/ml phenol in PBS. Under this precipitation condition, the concentration of insulinotropin remaining in solution was 0.18 mg/ml. When the phenol concentration was changed to 1.0 mg/ml, data identical to those in Figures 2 and 3 were obtained. However, a phenol concentration of 2.2 mg/ml was chosen for further study due to its antimicrobial preservative effectiveness.

Insulinotropin was precipitated in the presence of protamine and zinc. The effect of zinc on the isophane ratio was studied. Protamine was added at different zinc/insulinotropin molar ratios (Zn/Ins = 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, and 3.0). The data are shown in Figure 4. The total protein concentrations in the supernatant were significantly reduced by a combination of zinc and protamine. Additionally, the isophane ratio was affected by the zinc concentration.

In Figure 5, the isophane ratio is plotted as a function of Zn/Ins ratio. The isophane ratio increased with Zn/Ins ratio.

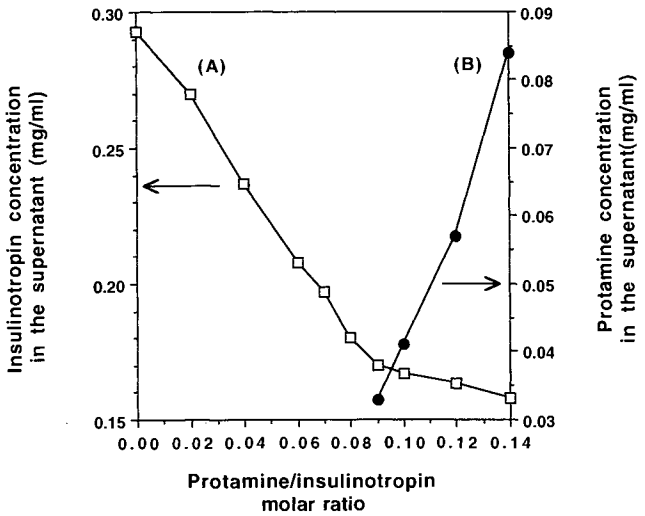

Fig. 2. The effect of protamine/insulinotropin molar ratio on insulinotropin and protamine concentrations in the supernatant in the presence of 2.2 mg/ml phenol.

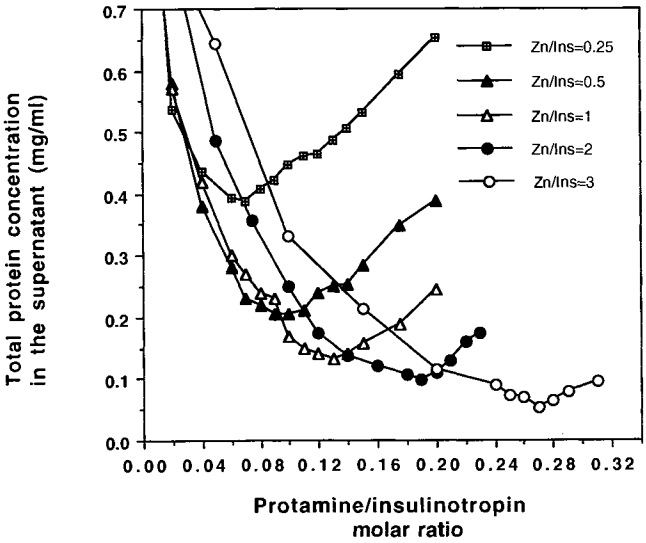

Fig. 4. The effect of zinc concentration on the total protein concentration in the supernatant. The data obtained with Zn/Ins = 0.1 and 1.5 are not shown in this figure.

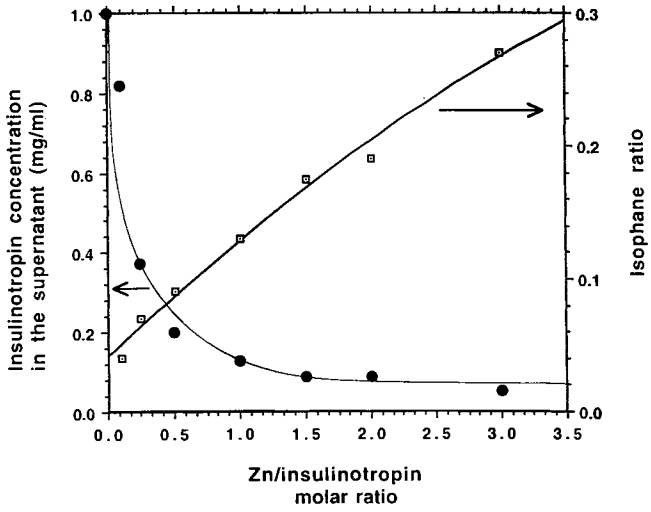

Fig. 5. The effect of zinc concentration on the isophane ratio (square) and the insulinotropin concentration (solid circle) in the supernatant.

For example, as the Zn/Ins ratio increased from 1 to 3, the isophane ratio doubled. In other words, as more zinc was added, more protamine was needed for the completion of precipitation.

When zinc was added to a protamine solution in PBS, it was found that the protamine precipitated. The amount of protamine precipitating with only zinc in the presence of both insulinotropin and zinc was not determined. However, some of the protamine may not have been available for insulinotropin precipitation due to the protamine/zinc precipitation, especially at higher zinc concentrations, because the isophane ratio increased with Zn/Ins ratio. This is evident when the protamine and insulinotropin concentrations in the supernatant are plotted separately as a function of Zn/Ins ratio at a constant Prt/Ins molar ratio. Figure 6 illustrates this for a Pro/Ins ratio of 0.1. As the Zn/Ins ratio increased from 0 to 1.0, the concentration of both proteins in the supernatant decreased. On the contrary, when the Zn/Ins ratio exceeded 1.0, the insulinotropin concentration in the supernatant increased with increasing Zn/Ins ratio and the protamine was not detected in the supernatant. This suggests that the protamine is less available for insulinotropin at a higher Zn/Ins ratio than 1.0.

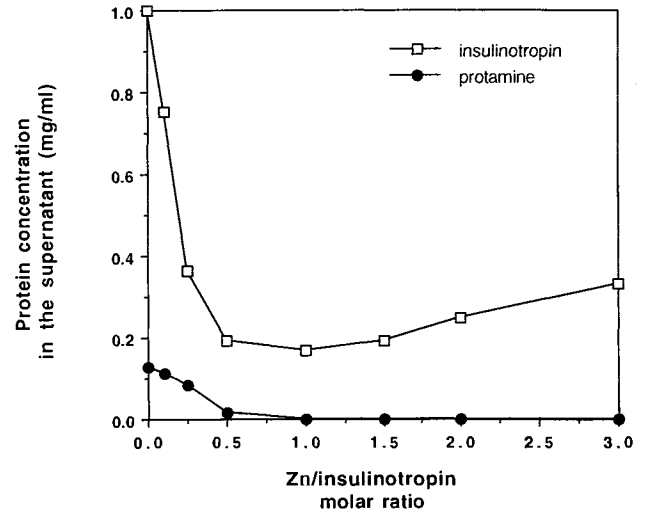

Fig. 6. The effect of zinc concentration on the insulinotropin and protamine concentrations in the supernatant at the protamine/insulinotropin molar ratio of 0.1.

The insulinotropin concentration in the supernatant at each isophane ratio are also shown in Figure 5. When the Zn/Ins ratio increased from 0 to 1.0, the insulinotropin concentration in the supernatant showed a rapid decrease from 1.0 to 0.130 mg/ml. Increasing the Zn/Ins ratio beyond 1.0 decreased the insulinotropin concentration more slowly; at a 3:1 ratio, the concentration was 0.053 mg/ml. However, the decrease in insulinotropin concentration from 0.130 mg/ml to 0.053 mg/ml was accompanied by a significant increase in isophane ratio; in other words, an increase in protamine concentration from 0.17 to 0.34 mg/ml. Therefore, a 1:1 Zn/Ins ratio is the best ratio to obtain both low insulinotropin concentration in the supernatant and low amount of protamine.

A possible synergistic effect between phenol and zinc on the precipitation of insulinotropin in the presence of protamine was investigated. Both phenol and zinc were used as additives for the precipitation of insulinotropin in the presence of protamine. The phenol concentration was kept constant at 2.2 mg/ml, the concentrations of zinc and protamine were changed, and the protein concentrations in the supernatant were monitored. An increase in isophane ratio with an increase in zinc concentration was observed also in this experiment. Zn/Ins ratio of 0.5, 1.0, and 1.5 gave isophane ratios of 0.08, 0.11, and 0.14, respectively. The minimum insulinotropin concentration of 1 μg/ml in the supernatant was found with a 1:1 Zn/Ins ratio. A further reduction of the insulinotropin concentration in the supernatant could not be achieved by adjusting zinc and/or protamine concentration.

In conclusion, we developed a method to determine the isophane ratio of protamine/insulinotropin precipitation using HPLC. This method was used to estimate the minimum quantity of protamine required for the completion of protamine/insulinotropin precipitation in the presence of different excipients. This approach can be used for any other basic/acidic protein precipitation for which analytical HPLC methods can be established.

ACKNOWLEDGMENTS

We would like to express our thanks to Dr. Wayne A. Boettner and Dr. Timothy A. Hagen who made helpful comments on the manuscript. We thank Mr. Richard A. Grucza for the CD measurement, Mr. Dennis Danley for the isoelectric focussing experiment, and Dr. Cheryl M. Kirkman for the HPLC method development for insulinotropin assay.

REFERENCES

1. J. A. Hoffmann, R. E. Chance, and M. G. Johnson. Purification and analysis of the major components of chum salmon protamine contained in insulin formulations using HPLC. *Protein Expression and Purification* 1:127–133 (1990).
2. J. M. Weiler, P. Freiman, M. D. Sharath, W. J. Metzger, J. M. Smith, H. B. Richerson, J. K. Ballas, P. C. Halverson, D. J. Shulan, S. Matsuo, and R. L. Wilson. Serious adverse reactions to protamine sulfate: are alternatives needed? *J. Allergy Clin. Immunol.* 75:297–303 (1985).

3. R. H. Dreisbach. *Handbook of Poisoning: Diagnosis and Treatment*. Lange Medical Publications, Los Altos, CA, 1971, pp. 331–334.
4. P. Felig. Protamine insulin: Hagedorn's pioneering contribution to drug delivery in the management of diabetes. *J. Am. Med. Assoc.* **251**:393–396 (1984).
5. J. B. Martin, L. P. Renaud, and P. Brazeau. Pulsatile growth hormone secretion: suppression by hypothalamic ventromedial lesions and by long-acting somatostatin. *Science* **186**:538–540 (1974).
6. P. Brazeau, J. Rivier, W. Vale, and R. Guillemin. Inhibition of growth hormone secretion in the rat by synthetic somatostatin. *Endocrinology* **94**:184–187 (1974).
7. G. S. Tannenbaum and E. Colle. Ineffectiveness of protamine zinc somatostatin as a long-acting inhibitor of insulin and growth hormone secretion. *Can. J. Physiol. Pharmacol.* **58**:951–955 (1980).
8. J. Buch and A. Buch. Sustained effect of zinc-protamine-glucagon in hyperlipidaemic patients. *Acta Pharmacol. Toxicol.* **53**:188–192 (1983).
9. J. P. Naets and M Guns. Inhibitory effect of glucagon on erythropoiesis. *Blood* **55**:997–1002 (1980).
10. H. C. Hagedorn. The absorption of protamine insulin. *Rep. Steno. Hosp. (Cph.)* **1**:25–28 (1946).
11. H. C. Hagedorn, B. N. Jensen, N. B. Krarup, and I. Wodstrup. Protamine insulinate. *J. Am. Med. Assoc.* **106**:177–180 (1936).
12. H. O. Bang. Some investigations on the absorption mechanism of protamine insulin. *Acta Pharmacol Toxicol (Copenh)* **2**:79–88 (1946).
13. K. Brunfeld and J. E. Poulsen. Protamine-splitting enzyme from serum and subcutaneous tissue. *Rep. Steno. Mem. Hosp. Nord. Insulinlab* **5**:51–61 (1953).
14. R. N. Shore, W. B. Shelley, and G. C. Kyle. Chronic urticaria from isophane insulin therapy. Sensitivity associated with non-insulin components in commercial preparations. *Arch Dermatol* **111**:94–97 (1975).
15. M. B. Sanchez, M. Paolillo, R. S. Chacon, and M. Camejo. Protamine as a cause of generalized allergic reactions to NPH insulin. *Lancet* **I**:1243 (1982).
16. M. E. Weiss, F. Chatham, A. Kagey-Sobotka, and N. F. Adkinson, Jr. Serial immunological investigations in a patient who had a life-threatening reaction to intravenous protamine. *Clin. Exp. Allergy* **20**(6):713–720 (1990).
17. J. A. Ellerhorst, J. P. Comstock, and L. J. Nell. Protamine antibody production in diabetic subjects treated with NPH insulin. *Am. J. Med. Sci.* **299**(5):298–301 (1990).
18. L. J. Nell and J. W. Thomas. Frequency and specificity of protamine antibodies in diabetic and control subjects. *Diabetes* **37**:172–176 (1988).
19. J. C. Horrow. Protamine: a review of its toxicity. *Anesth. Analg.* **64**:348–361 (1985).
20. H. C. Hagedorn. Fortschritte in der insulin-therapie. *Schweiz. Med. Wchnschr.* **68**:37–41 (1938).
21. J. Brange. *Galenics of Insluin: The Physicao-chemical and Pharmaceutical Aspects of Insulin and Insulin Preparations*. Springer-Verlag, New-York, 1987, pp. 43–44.
22. R. D. Simkin, S. A. Cole, H. Ozawa, B. Magdoff-Fairchild, P. Eggena, A. Rudko, and B. W. Low. Precipitation and crystallization of insulin in the presence of lysozyme and salmine. *Biochim. Biophys. Acta* **200**:385–394 (1970).
23. G. I. Bell, R. Sanchez-Pescador, P. J. Laybourn, and R. C. Najarian. Hamster preglucagon contains the sequence of glucagon and two related peptides. *Nature (Lond.)* **302**:716–718 (1983).
24. G. C. Weir, S. Mojsov, G. K. Hendrick, and J. F. Habener. Glucagonlike peptide I (7–37) actions on endocrine pancreas. *Diabetes* **38**:338–342 (1989).
25. L. C. Lopez, M. L. Frazier, C. J. Su, A. Humar, and G. F. Sanders. Mammalian pancreatic preproglucagon contains three glucagon-related peptides. *Proc. Natl. Acad. Sci. U.S.A.* **80**:5485–5489 (1983).
26. S. Seino, M. Welsh, G. I. Bell, and F. Steiner. Mutations in the guinea pig preproglucagon gene are restricted to a specific portion of the prohormone sequence. *FEBS Lett.* **203**:25–30 (1986).
27. W. W. Day, M. West, Y. Kim, and M. R. Nocerini. The effect of protamine and zinc excipients on the pharmacokinetics of subcutaneous insulinotropin formations in male rats. AAPS National Meeting 1994, San Diego, CA.
28. W. W. Day and Y. Kim. The effect of protamine, phenol, and zinc on the pharmacokinetics of insulinotropin suspensions in male rats after subcutaneous injections. Manuscript in preparation.
29. J. Brange and L. Langkjær. Chemical stability of insulin 3. Influence of excipients, formulation, and pH. *Acta Pharm. Nord.* **4**(3):149–158 (1992).
30. D. T. Graham and A. R. Pomeroy. An in-vitro test for the duration of action of insulin suspensions. *J. Pharm. Pharmacol.* **36**:427–430 (1984).