Insulin Precipitation in Artificial Infusion Devices

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Summary. Precipitation of insulin is a problem with mechanical insulin infusion devices. Therefore conditions affecting insulin precipitation have been studied in a recirculating system using a peristaltic pump. Acetate buffer favoured precipitation and a pH of 2, 3 or 8 resulted in no less (and often more) precipitation than a pH of 7 using either acetate or phosphate buffer. However, no precipitation occurred in a phosphate buffered neutral preparation after ten days of pumping through tubing pretreated with EDTA. In comparison at pH 7, using acetate buffer with untreated tubing, more than 95% of insulin was precipitated in ten days. A heavy metal-insulin association appears to be a major factor in the precipitation. However, some formation of insulin fibrils is probable as the precipitate is autocatalytic and partially acid insoluble. Phosphate buffered highly purified porcine insulin is suitable for relatively prolonged infusion if metal ion contamination of the delivery system is minimised.

Key words: Insulin/administration and dosage/therapeutic use, infusion devices, EDTA, phosphate buffered insulin, acetate buffered insulin.

Open-loop insulin infusion devices under clinical investigation for diabetes therapy vary from simple syringe pumps used for subcutaneous infusion [1–3] to more sophisticated devices used principally for intravenous infusion [4, 5]. In addition, devices for implantation are being developed [6]. A problem now recognised by several groups [4–6] which limits further development of these devices is the tendency of insulin to form insoluble precipitates in pumping systems. Although the mechanism and nature of insulin aggregation or polymer formation under static conditions

is well described [for review 7] the nature of insulin precipitation during continuous infusion is poorly understood. In a recent review [8] insulin precipitation or aggregation was described as the major impediment to improvement of artificial insulin delivery systems. Insulin precipitation may cause obstruction of flow in pump tubing [4] as well as a reduction in the biological potency of the infusate [9]. The problem appears to relate to pump design. No precipitation has been reported [1–3] or observed by us using syringe based systems where insulin is replaced every 1–2 days. However, the problem is significant where insulin is pumped for longer periods through small orifices [6] or where a peristaltic system is used [5].

The aim of this study was to define conditions under which clinical insulin preparations can be infused without significant precipitation. The relative suitability for infusions of up to ten days, seen as the projected maximal time between reservoir exchange in the next generation of infusion devices, was investigated. A recirculating peristaltic system was chosen to exaggerate any problems of insulin precipitation. The pH has been previously reported both to affect [5] or not affect [6] insulin precipitation. Also metal ions have been reported to influence insulin precipitation [8]. With these reports in mind an assessment was made of two buffers commonly used in neutral insulin preparations under conditions of varying pH and metal ion concentration.

Materials and Methods

The following highly purified porcine insulin preparations were studied: neutral soluble insulin BP, acetate buffered pH 7 (Actrapid 80 units/ml, Novo); neutral soluble insulin USP, unbuffered pH 7 (Actrapid, 100 units/ml, Novo); soluble insulin BP, unbuffered pH 3 (80 units/ml, Novo) and neutral soluble insulin, phosphate buffered pH 7 (Leo Neutral, 80 units/ml, Nordisk).

Fig. 1. Fractionation on Sephadex G-50 fine of neutral phosphate buffered highly purified porcine insulin after 10 days pumping in a recirculating peristaltic pumping system. Comparison with a similar unpumped control preparation. ●—● = pumped, ○—○ = control. The column was calibrated with blue dextran (V_0), ^{125}I -human prolactin (mol.wt. 21,500) and $^{57}\text{CoCl}$

Table 1. Insulin precipitation after 10 days recirculation expressed as a percentage of protein content (method of Lowry et al. [10]). All solutions contained highly purified porcine insulin (see Methods)

Buffer	Protein precipitated (%)			
	Untreated tubing		EDTA treated tubing	
	Neutral (pH = 7)	Neutral (pH = 7)	Acid	Alkaline (pH = 8)
Phosphate	48	0	70 (pH 2)	8.3
Acetate	100	74	95 (pH 2)	73
None	93	57	10 (pH 3)	NT

NT = not tested

Using aseptic techniques insulin preparations (2 ml) were pumped at a rate of 0.9 ml/h from rubber stoppered glass reservoirs connected by 22 gauge stainless steel needles through 40 cm silicone rubber tubing 0.02 inches inside diameter $\times$ 0.08 inches outside diameter (Manostat, New York). Insulin solutions were recirculated in the system at ambient temperature (20 °C–25 °C) by a Desaga peristaltic pump. Unless stated otherwise the tubing was not treated before use. In the case of EDTA treatment, segments of tubing were boiled in 10 mmol/l EDTA for 1 h followed by further boiling for 1 h in deionised water. Prior to use, 20 ml deionised water was pumped through each segment of tubing. In examining the effects of pH, the various preparations were adjusted with NaOH (1 mmol/l) or HCl (1 mmol/l) to the desired pH. Ten days of recirculation did not affect the pH of any preparation.

To quantitate the degree of precipitation, precipitates were resuspended and 0.5 ml aliquots from each glass reservoir were centrifuged at 8,000 g for 5 min. The precipitates were washed twice with 1 ml distilled water and dissolved in 0.5 ml NaOH (1 mmol/l). Protein concentration was measured by the method of Lowry et al. [10] using bovine serum albumin as standard. To establish whether insulin aggregates were formed in the absence of a precipitate insulin was fractionated on a 0.9 $\times$ 40 cm column of Sephadex G-50 fine equilibrated in 0.1 mmol/l sodium phosphate buffer (pH 7) at

4 °C with a flow rate of 18 ml/h. Columns were calibrated with blue dextran, ^{125}I -human prolactin (mol.wt. 21,500) and $^{57}\text{CoCl}$. Insulin in the column fractions was measured spectrophotometrically at 280 nm.

Immunoreactive insulin in the supernatants and column fractions was assayed by a double antibody radioimmunoassay [11] using ^{125}I -human insulin and human insulin as standard.

Further experiments were performed in which (i) a precipitate 'seed' was prepared by redispersing a small amount of previously obtained precipitate in phosphate buffer; 0.1 ml of this solution ($< 0.4 \mu\text{g}$ protein) was added to a fresh acetate buffered insulin solution and pumped as described previously; (ii) acid solubility of washed precipitates was examined by resuspension of the pellet in 1N HCl and incubation.

Results

The effects of diluent (buffer composition and pH) and EDTA treatment of silicone rubber tubing on insulin precipitation after 10 days of pumping in the recirculating system at ambient temperature are shown in Table 1. Using untreated tubing precipitation occurred in phosphate, acetate and unbuffered preparations and was visibly evident from day 3 of pumping.

Pretreatment of tubing with 10 mmol/l EDTA considerably reduced the degree of precipitation at neutral pH in all preparations and in fact abolished precipitation in the phosphate buffered preparation. In a control experiment, boiling the tubing in water alone had no effect on the degree of precipitation. Adjustment of the pH of either the phosphate or acetate buffered preparation to 2 or 8 failed to reduce the degree of precipitation compared with the respective neutral preparation. In fact these pH adjustments mostly resulted in increased precipitation (Table 1). There was a moderate degree of precipitation (10%) in the acid unbuffered preparation.

The acetate buffered, neutral pH solution pumped for 10 days precipitated markedly; the protein content of the precipitate obtained using untreated tubing accounted for all of the protein measured in the unpumped control solution (2.8 mg/ml using a BSA standard) within the precision of the assay. This agreed well with the loss of insulin immunoreactivity from the supernatant which was greater than 95%.

The phosphate buffered neutral pH preparation which showed no precipitation following EDTA treatment of the tubing was found to be indistinguishable after the 10 day study from an unpumped control insulin solution as determined by gel filtration on Sephadex G-50 (Fig. 1). The fractions were assayed by absorption spectrophotometry at 280 nm (Fig. 1). Serial dilution of the peak fractions resulted in recovery of the expected levels of insulin immunoreactivity. Parallel dilution curves were obtained for pumped and control insulin solutions.

The addition of acetate to the neutral phosphate buffered preparation resulted in precipitation (73%) equivalent to that of the acetate buffered preparations alone (Table 1).

Results reported in Table 1 were obtained from experiments using the same batch of silicone rubber tubing. Considerable variations were found in the extent of precipitation seen with different batches of tubing. However, the same relative stability was observed; that is, in seven trials on four different batches of tubing the acetate buffered preparation precipitated markedly six times using both EDTA treated and untreated tubing, whereas no precipitation was observed in any of the seven trials with the phosphate buffered preparation and EDTA treated tubing.

The addition of an insoluble seed (see Methods) to an acetate buffered insulin solution resulted in marked precipitation (80% as determined by the method of Lowry et al. [10]) within two days. A concurrently pumped control solution did not precipitate until seven days. This indicated that precipitate formation can be autocatalytic.

When precipitates were treated with acid most of the insulin was solubilised (70%–80% in acid supernatant). However, a small fraction was not recovered in the acid supernatant. The remaining precipitate was dissolved in 1N NaOH and assayed for insulin. Although immunological activity was detected, accurate quantitation was not possible as this preparation did not dilute parallel to a porcine insulin standard.

Discussion

Insulin precipitation poses a serious problem in the development of devices for long-term continuous insulin infusion. Previous attempts to obtain a diluent suitable for pumping insulin without precipitation have been unsuccessful [6]. Serum has been used as an additive to reduce precipitation [9], but would have obvious disadvantages unless the active component can be characterised.

In this study an analysis has been made of simple modifications to insulin solutions and pump tubing in an effort to define conditions suitable for insulin infusion without precipitation where the reservoir and pump tubing were used continuously for ten days. There was a considerable variation in stability of insulin preparations tested and only the neutral, highly purified phosphate-buffered preparation remained totally in solution after ten days of pumping. It was, however, necessary to pretreat tubing with EDTA to obtain this absolute degree of stability. There was then no change in apparent molecular weight or insu-

lin immunoreactivity of this insulin preparation during pumping (Fig. 1).

The major effect of EDTA treatment is presumably removal from the tubing of divalent cation contaminants, the presence of which would be likely to contribute to insulin precipitation. Insulin will aggregate to form the hexamer not only in the presence of zinc, but also in the presence of copper, manganese, iron and nickel [7]. The zinc-insulin hexamer is predominant in the neutral pH range and is stable and soluble up to a zinc ion concentration of two per hexamer. As the ratio increases, firstly a 72,000 Dalton form occurs while above 6 zinc ions per hexamer multimers of insulin 200–300,000 Daltons can be found [7].

Our data suggest that this mechanism is responsible for a large fraction of the observed insulin precipitation. Acetate ions, which are known to increase the incorporation of zinc into insulin hexamers (personal communication, G. Dodson) enhance precipitation. Also, EDTA, an agent which chelates and solubilises heavy metal ions, decreases precipitation. However, there is further evidence to suggest that alternative mechanisms may contribute to precipitation. Firstly, at acid pH, zinc is not bound to the insulin hexamer and dimers predominate [7]. In contrast to our results (Table 1), precipitation should not occur at acid pH since large molecular weight zinc-insulin polymers are not formed [7]. Secondly, the amount of zinc-insulin polymer formed is dependent on the amount of zinc available in solution. Therefore, precipitation by this mechanism should not proceed autocatalytically. This, however, is in contrast to the results of the seeding experiment.

Thirdly, zinc-insulin polymers are soluble at acid pH due to the acid dependent dissociation of zinc from the polymer. While approximately 80% of the insulin in the precipitate, obtained from an acetate buffered preparation, could be recovered by acid treatment, the remainder was soluble only above pH 10.

It is possible that the acid-insoluble component of the precipitate represented insulin fibril formation. Insulin fibrils are biologically inactive long-chain polymers of insulin [12] whose formation is favoured by acid pH [13]. They elongate rapidly by an autocatalytic mechanism, removing native insulin from solution [13].

Our studies are relevant to the development of insulin delivery devices. Using simple syringe infusers [1–3] the insulin reservoir (syringe) is replaced every 1–2 days and precipitation is not a problem. However, for the next generation of devices, incorporating reservoirs with up to ten days of supply of insulin, conditions of infusion and the choice of insulin preparation will be critical. We suggest that firstly precau-

tions be taken to minimise metal ion concentrations and, secondly, in view of the autocatalytic aspect of the precipitation, that exchangeable reservoirs be preferred. A phosphate buffered neutral highly purified insulin preparation is compatible with the next stage of device development.

Acknowledgements. This work has been supported by the National Health and Medical Research Council of Australia. We wish to thank Dr. Guy Dodson, University of York and Drs. Norman Coles and Len Hartman of Commonwealth Serum Laboratories, Melbourne, for helpful comments. We wish also to thank Mr. John Miller, of Novo Laboratories, Sydney, for arranging supply of USP insulin and supplying information on clinical insulin preparations.

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Received: 9 February 1981
and in revised form: 22 June 1981

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