Reduced Solubility of Short-Acting Soluble Insulins when Mixed with Longer-Acting Insulins

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SUMMARY

Using insulins from three manufacturers, we examined the recovery by radioimmunoassay of short-acting soluble insulin when mixed with long-acting insulin as a function of the ratio of the mixture and the time of premixing. In ratios of 1:2, 1:3, and 1:5 (short- to long-acting insulin), all Novo, Nordisk, and Lilly short-acting insulins tested showed a significant loss of solubility when mixed with the respective company's long-acting insulin either for less than 75 s or for 20 min before centrifugation.

In ratios of 1:1, Novo's Actrapid (regular) with Monotard (lente) and Lilly's regular with lente showed no significant loss of solubility when pre-mixed for less than 75 s, and the regular insulin also showed no significant loss when pre-mixed for 20 min. However, when Lilly's regular was mixed with either NPH or ultralente in a 1:1 ratio, a significant loss of solubility of the short-acting insulin occurred regardless of time [as was also found with Nordisk's Velosulin (regular) with Insulatard (NPH)].

When Lilly regular was incubated with Lilly lente in ratios of 1:3 for <75 s, 20 min, 4 h, and 24 h before centrifugation, there was a progressive loss of solubility. In contrast, with the same ratios and times of premixing, Lilly regular when mixed with Lilly NPH showed a rapid initial loss of solubility that plateaued by 20 min before centrifugation.

Supernatants from the centrifugation of Lilly's long-acting insulins were assayed and found to contain only 0.67–1.5% of the total insulin content. When Lilly regular was mixed in a 1:3 ratio with these supernatants, there was a small but significant loss of solubility.

These in vitro data indicate that short-acting insulins may lose solubility when mixed with long-acting insulins as the proportion of the latter increases and the time of pre-mixing is prolonged. **DIABETES *32*:1177–1181, December 1983.**

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Mixtures of short- and long-acting insulins, injected subcutaneously, remain the most widely used therapy in patients with insulin-dependent diabetes.[1,2] The theoretical advantage of injecting an insulin mixture before breakfast and dinner is that the soluble insulin will be rapidly absorbed, acting within 15–30 min to meet the immediate requirements during the absorptive phase, while the longer-acting insulin will provide coverage for the remainder of the day and night.

The use of insulin mixtures is predicated upon the assumption that the absorption kinetics of the soluble short-acting insulin are not influenced by mixing in the same syringe with insoluble longer-acting insulin. This is generally unquestioned in most textbooks and in the literature distributed by pharmaceutical companies.[3] Recently, however, several investigators have reported in vivo data that raise concern about the clinical bioavailability of soluble insulin when mixed with longer-acting insulins in certain ratios and for different times before injection.[2,4–6]

The methods used to prolong the action of insulin involve its precipitation either with a protein substance (protamine) or in an acetate buffer at a particular concentration of zinc ions (lente series), and it is possible that these residual materials influence the solubility of admixed short-acting insulin. To examine this question, we measured the recovery of soluble insulin when mixed in varying ratios with long-acting insulin suspensions for different periods of pre-mixing.

MATERIALS

At least 36 different vials from diverse lots of highly purified porcine U-100 insulins were used. These included three soluble short-acting preparations: Iletin II regular (Eli Lilly, Indianapolis, Indiana), Actrapid (Novo, Copenhagen, Denmark), and Velosulin (Nordisk, Copenhagen, Denmark). These were mixed in varying ratios with a longer-acting in-

sulin suspension from the same manufacturer: Iletin II regular with Iletin II lente, NPH, or ultralente (Eli Lilly); Actrapid with Monotard (a lente-like suspension of porcine insulin from Novo); and Velosulin with Insulatard (NPH insulin from Nordisk). Dilutions were made in 0.4 M glycine containing 1% albumin at pH 8. All procedures were done with plastic items, including pipettes, test tubes, and syringes. Pipetting was done with a Gilson Pipetman (Middleton, Wisconsin) automatic pipette.

METHODS

The recovery of soluble short-acting insulin after mixing for different time periods with a longer-acting insulin suspension or its supernatant was determined by radioimmunoassay.[7] The supernatants of long-acting insulins were also assayed for any soluble insulin. Results are expressed as the mean ± SEM. The following four protocols were used:

I. Pre-mixing of insulins for a mean time of 20 min. The entire contents of bottles of short- and long-acting insulins from each manufacturer were transferred to test tubes. Short-acting insulin (0.5–1.0 ml) was pipetted into other tubes, and longer-acting insulin suspensions were rapidly added after thorough dispersal of particles to make 1:1, 1:2, and 1:3 (short- to long-acting ratios). A mixture of regular insulin (0.5–1.0 ml) with 0.4 M glycine and 1% bovine albumin buffer in the same ratios served as the control. Preparation of the insulin mixtures involved a mean time of 20 min before centrifugation (range:10–30 min).

The insulin mixtures were vortexed for 1–2 s and then centrifuged at 1000 × *g* for 20 min.

The supernatant (0.5 ml) was withdrawn from each sample, diluted in glycine-albumin buffer (1:100), stored at −20°C, and subsequently assayed for insulin levels by radioimmunoassay.

II. Incubation of insulins for less than 75 s. To determine if the loss of short-acting insulin is time-dependent, Lilly, Novo, and Nordisk short- and long-acting insulins were in contact for less than 75 s before centrifugation. (The rapid handling of the specimens was accomplished by mixing only three or four samples at a time.) The procedure was identical to that described above except that (1) the insulin mixtures were not vortexed (to simulate the clinical situation) but were centrifuged immediately after longer-acting insulin was added; (2) 1:1, 1:3, and 1:5 ratios were tested; and (3) Lilly ultralente was not tested in the 1:5 ratio.

III. Incubation of Lilly insulins for 4 and 24 h. To determine if the loss of short-acting insulin is a function of the time of incubation, Lilly regular was pre-mixed with Lilly lente and Lilly NPH in a ratio of 1:3 for 4 and 24 h before centrifugation. The procedure was identical to that described in protocol I.

IV. Incubation of short-acting insulins with the supernatants of longer-acting insulins for a mean time of 20 min. To compare the effects of the supernatants alone versus the intact suspension of longer-acting insulin on the solubility of admixed regular insulin, the following experiment was performed. The entire contents of bottles of Iletin II lente, NPH, and ultralente were transferred to test tubes, then centrifuged at 1000 × *g* for 20 min. Iletin II regular was then combined with the supernatants in a 1:3 ratio, incubated for a mean time of 20 min, and recentrifuged. Regular insulin mixed with glycine-albumin buffer served as the control. The samples were then processed for assay as described in protocol I.

In addition, aliquots from the supernatants of the long-acting insulins were diluted in glycine-albumin buffer and assayed for immunoreactive insulin before mixing with regular.

Calculations and statistical analysis. The recovery of short-acting insulin is expressed as the ratio of the actual recovery (by radioimmunoassay) over the expected recovery times 100 (mean percent recovery ± SEM). The percentage of short-acting insulin recovered in the control was statistically compared (by Student's *t* test) with that in the mixture for each manufacturer at each ratio.

RESULTS

Recovery of soluble short-acting insulin after mixing with longer-acting insulin suspensions for less than 75 s (Table 1). When Novo Actrapid and Monotard and Lilly regular and lente were mixed in a 1:1 ratio and then immediately centrifuged, there was no significant loss of the Actrapid or

TABLE 1
Recovery of soluble short-acting insulin after mixing in varying ratios with long-acting insulin suspensions for a mean time of less than 75 s and for 20 min

Ratio (short:long)	Manufacturer	Recovery of short-acting insulin (%)							
		<75 s				20 min			
		Control*	Lente	NPH	Ultralente	Control	Lente	NPH	Ultralente
1:1	Lilly	102 ± 1.2(12)†	96 ± 2.7 (12)	75 ± 1.7‡(4)	90 ± 2.7‡(4)	105 ± 2.5(16)	100 ± 1.9 (12)	72 ± 1.3‡(12)	93 ± 2.2‡(14)
	Novo	106 ± 2.4(8)	102 ± 5.0 (8)			99 ± 0.7(7)	87 ± 1.6‡(7)		
	Nordisk	106 ± 3.9(8)		77 ± 2.0‡(8)		97 ± 1.9(7)		73 ± 1.0‡(7)	
1:2	Lilly					103 ± 1.9(11)	81 ± 1.1‡(7)	46 ± 1.6‡(7)	64 ± 1.3‡(9)
	Novo					100 ± 2.7(7)	50 ± 1.2‡(7)		
	Nordisk					100 ± 3.1(12)		63 ± 1.6‡(12)	
1:3	Lilly	101 ± 2.2(4)	74 ± 2.1‡(4)	42 ± 1.7‡(4)	69 ± 0.3‡(4)	110 ± 2.2(11)	53 ± 1.4‡(10)	24 ± 1.7‡(11)	37 ± 2.4‡(13)
	Novo	106 ± 6.3(8)	49 ± 1.9‡(8)			101 ± 2.7(7)	29 ± 1.2‡(7)		
	Nordisk	105 ± 4.4(4)		62 ± 1.8‡(4)		99 ± 2.8(12)		43 ± 1.2‡(12)	
1:5	Lilly	89 ± 2.2(4)	38 ± 0.6‡(4)	30 ± 0.4‡(4)					
	Novo	97 ± 1.5(4)	25 ± 1.3‡(4)						
	Nordisk	93 ± 1.3(4)		25 ± 0.5‡(4)					

*0.4 M glycine/1% bovine albumin buffer.
†Numbers in parentheses indicate number of samples.
‡$P < 0.001$ versus control.

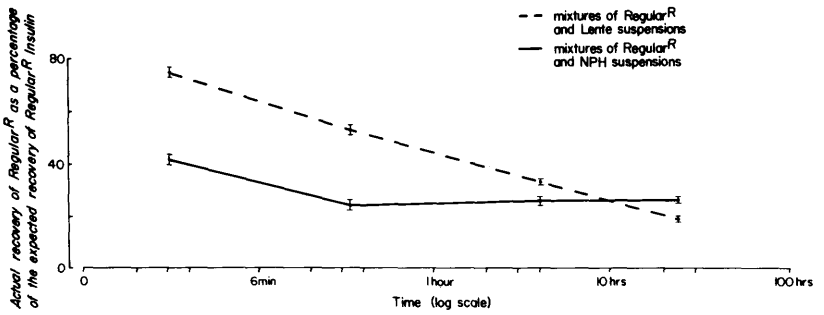

FIGURE 1. Mean recovery of Iletin II regular insulin after pre-mixing (1:3 ratio) with suspensions of Iletin II lente and NPH for less than 75 s and for 20 min, 4 h, and 24 h. The statistical significance of the percentage of regular insulin recovered in the mixtures, as compared with the controls, was $P < 0.001$ for all samples.

regular insulin, although there was a large and significant loss in the 1:3 and 1:5 ratios. In contrast, there was a significant loss of Velosulin and Lilly regular in the 1:1, 1:3, and 1:5 ratios when mixed with Insulatard and Lilly NPH or ultralente, respectively.

Recovery of short-acting insulin after mixing with varying ratios of longer-acting insulin suspensions for a mean time of 20 min (see Table 1). When Lilly regular insulin was mixed with Lilly lente in a 1:1 ratio, there was no significant difference in the mean percentage recovered vis-à-vis the control. In contrast, there was a small but significant reduction in the recovery of Lilly regular insulin after mixing with suspensions of Lilly NPH (72 ± 1.370% reduction) and ultralente (93 ± 2.2% reduction). Similarly, a reduction in recovery of short-acting insulin was noted when Actrapid was mixed with Monotard (Novo) (87 ± 1.6% reduction) and Velosulin with Insulatard (Nordisk) (73 ± 1.070% reduction) in 1:1 ratios.

All three manufacturers' short-acting insulins showed a more striking reduction in the percentage recovered when mixed in 1:2 and 1:3 ratios with their respective lente, NPH, and ultralente insulins.

The results demonstrate that, as the proportion of longer-acting insulin added to the mixture increases, the recovery of the soluble short-acting insulin decreases. As with the shorter incubation period, more short-acting insulin was lost as the proportion of longer-acting insulin increased.

Recovery of Lilly regular insulin after mixing with Lilly lente and NPH (1:3 ratio) for 4 and 24 h. Prolonged incubation of Lilly regular insulin with Lilly lente insulin led to a significant progressive time-dependent loss of solubility of the regular insulin, with only 19.6 ± 0.2% being recovered by 24 h. Prolonged incubation of regular with Lilly NPH led to an initial significant loss of solubility that appeared to plateau (range: 25–26%) by 20-min incubation time (Figure 1).

Recovery of regular insulin after mixing with supernatants of longer-acting insulins. Pre-mixing Lilly regular in a 1:3 ratio with supernatants of Lilly lente, NPH, and ultralente insulins led to a minimal but significant loss; however, the

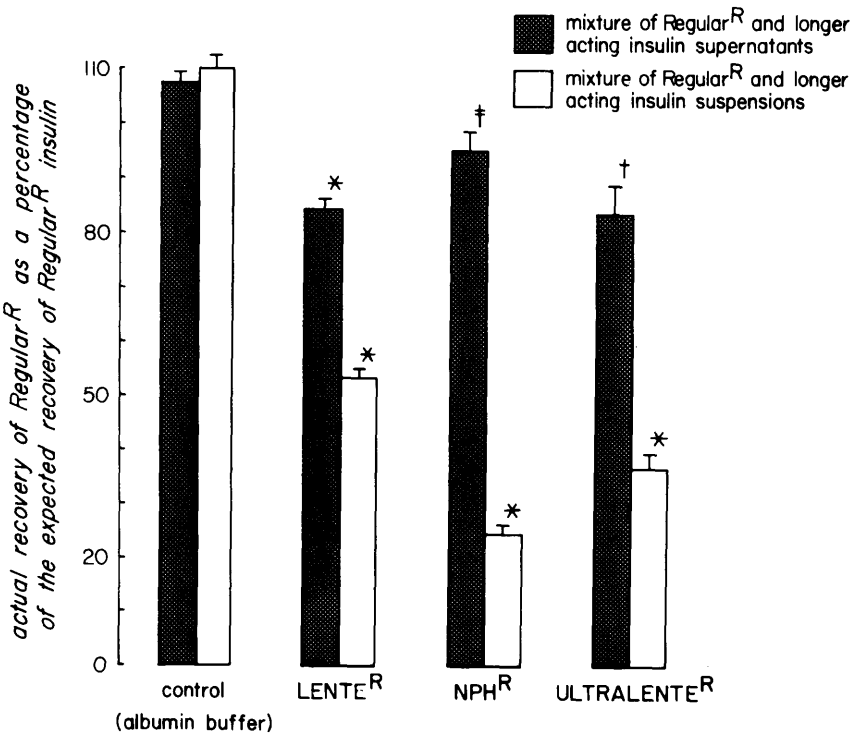

FIGURE 2. Mean recovery of Iletin II regular insulin after pre-mixing (1:3 ratio) with supernatants or suspensions of Iletin II lente, NPH, and ultralente insulins for a mean time of 20 min. The statistical significance of the percentage recovered in the mixtures, compared with controls, was: *$P < 0.001$, †$P < 0.005$, and ‡$P < 0.02$.

loss of the regular was much more profound when mixed with insulin suspensions (Figure 2).

The mean amounts of immunoreactive insulin detected in the supernatants were 0.72 ± 0.06 U/ml per bottle of Iletin II lente, 0.67 ± 0.12 U/ml per bottle of Iletin II NPH, and 1.5 ± 0.1 U/ml per bottle of Iletin II ultralente.

DISCUSSION

These in vitro results demonstrate that short-acting insulin loses solubility when mixed with longer-acting insulin and that this loss is a function of the ratio and the forms of insulin as well as of the duration of contact.

The mechanism of the observed loss of solubility of short-acting insulin in mixtures is still speculative. Our data show that, in longer-acting insulins, the particulate fraction plays the dominant role in facilitating precipitation. When short-acting insulin was mixed only with supernatants of insulin suspensions, the loss was minimal in comparison with that observed after mixing with intact insulin suspensions. The excess zinc in the lente insulins may form complexes with added short-acting insulin to reduce the latter's solubility in the acetate buffer,[6,8] although the type of bond is not known. Given the intrinsic difficulties of forming a zinc-insulin crystal, the admixed short-acting insulin is more likely to form an amorphous aggregate similar to semilente.

Although NPH insulin was originally manufactured to be free of excess protamine, recent evidence suggests that excess protamine is present. With high-performance liquid chromatography, a 15–20% excess of protamine was found in a 1:3 mixture of Lilly regular with NPH insulin;[9] thus, NPH preparations may have an excess of insulin-binding sites. The degree of protamine excess may depend upon the brand of NPH insulin used, as Nordisk's Mixtard has slightly less protamine than Lilly's NPH.[9] This may explain why we found the recovery of Nordisk Velosulin (regular) to be higher than Lilly's regular at ratios of 1:2 and 1:3 when mixed with the companies' respective NPH preparations. Added short-acting insulin may be bound to the protamine in the NPH, leading to reduced solubility.

These in vitro observations raise the clinical concern that there will be a diminished immediate effect and a decreased as well as delayed peak effect of the admixed short-acting insulin. With lente insulin mixtures the degree of soluble insulin loss may be very time dependent, as both our studies and those of others[9] have shown that regular insulin becomes more insoluble the longer the incubation period. In contrast, mixtures of short-acting insulins and NPH insulins may equilibrate more rapidly, as shown here with the maximal loss of solubility for this particular ratio being completed after only 20 min of incubation.

In vivo studies also indicate that the effectiveness of short-acting insulin may be decreased when administered in mixtures.[2,4,10] Galloway et al. have demonstrated a retardation in the time of onset of the peak insulin concentration and the times of maximal blood glucose response and blood insulin concentration as the ratio of regular to lente was moved from 3:1 to 1:3.[4] These workers also found that there was no significant difference in the serum insulin concentration and blood glucose response when 1:1 mixtures of regular with lente or NPH were given in the same syringe or as separate injections. This is consistent with our finding that a 1:1 ratio of short- to long-acting insulins causes a minimal or insignificant loss of solubility.

Berger et al.[2] found that mixing Actrapid and Monotard in a 10:16 ratio and waiting 5 min before injection resulted in delayed peak insulin levels and significantly lower serum insulin levels in comparison with simultaneous separate injection. However, when the same ratio was injected immediately after mixing and when the same ratio of Leo Regular (Nordisk) and Leo NPH (Nordisk) was injected immediately or after only a 1-min incubation period, there was no change in the insulin kinetics. Although we found a small loss of short-acting insulin when it was mixed with either Lilly or Nordisk NPH at a 1:1 ratio and centrifuged either immediately or after a 20-min incubation, this in vitro loss may be too small to be clinically significant. Alternatively, the short-acting insulin/protamine complex may be relatively unstable, dissociating rapidly after subcutaneous injection (in contrast to short-acting insulin/Monotard mixtures). A 1:2 or 1:3 ratio of the short-acting insulin to NPH or a longer incubation period may have demonstrated a clinically significant loss of solubility.

Schlichtkrull's experiments with 1:1 and 1:3 ratios of Actrapid and Monotard suggest that the 1:3 ratio leads to a delay in the peak insulin action and diminution in the estimated maximal insulin concentration when compared with separate injections.[10]

Insulin-dependent diabetics frequently pre-mix their short- and longer-acting insulins over a whole range of ratios. For those with a stable dosage, short-acting insulin is often added directly to the bottle of longer-acting insulin. With the therapeutic advent of multiple daily insulin injections, patients often draw up their insulin dose into a syringe and give themselves the injections hours later while away from home. Disabled patients may have insulin pre-mixed in syringes by visiting nurses for days or weeks.

Many variables are known to complicate the absorption and action of subcutaneously administered insulin; these include the site of injection,[11] exercise,[2,12] immunologic mechanisms,[13,14] and the quantity of long-acting insulin.[15] Our in vitro data and the limited clinical studies by others suggest that insulin mixtures that are pre-mixed or contain a preponderance of long-acting insulin contribute to the unpredictable clinical response to injection. Further in vivo studies of the onset of peak insulin action and the intensity of insulin's effect over the full range of therapeutic ratios after pre-mixing for varying times should lead to more effective diabetic management.

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