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A self-regulating insulin delivery system. I. Characterization of a synthetic glycosylated insulin derivative

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Summary

A derivatized insulin, succinyl amido phenyl-α-D-glycopyranoside insulin (SAPG-insulin) has been synthesized for use in a self-regulating insulin delivery system. The product was a heterogenous mixture of glycosylated insulins differing in their degree and site of substitution. These products were purified and separated using Con A affinity chromatography followed by fast protein liquid chromatography (FPLC) on an anion exchange column. The resultant 4 products were isolated and characterized by end group analysis, sugar group quantitation and isoelectric focusing. In addition, radioimmunoassay cross-reactivity and in vivo biological activity were assessed. The major peak was B-1 phenylalanine monosubstituted SAPG-insulin, which had a potency equivalent to native bovine insulin. This makes the purified fraction, B-1 phenylalanine monosubstituted SAPG-insulin, an ideal candidate for use in the self-regulating insulin delivery system. The method utilized in this work is also potentially useful in the modification of proteins or peptides for enhanced gastrointestinal absorption.

Introduction

A glucose-controlled insulin delivery system has been proposed by Brownlee and Cerami (1979), and developed in our laboratory (Jeong et al., 1984, 1985; Sato et al., 1984). The scheme is based on competitive binding between glucose and glycosylated insulin for carbohydrate specific binding sites on the plant lectin, concanavalin A (Con A). Several glycosylated insulins have been synthesized, and the details can be found elsewhere (Jeong et al., 1984). A system has been constructed in which Con A, immobilized to Sepharose beads and glycosylated insulin, are enclosed within a polymer membrane, through which glucose and glycosylated insulin can diffuse as shown in Fig. 1. Dynamic glucose influx results in the release and subsequent efflux of glycosylated insulin in proportion to the concentration of glucose.

The synthetic procedure to form the glycosylated insulins utilizes the mixed anhydride method which results in the formation of a mixture of insulin derivatives varying in their sites and degrees of substitution. These components may differ in their biological, physical and immunogenic properties; therefore, studies were un-

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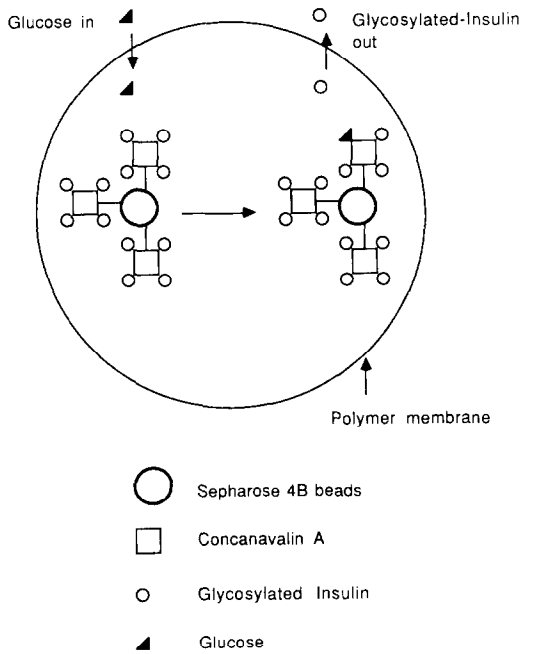

Fig. 1. A schematic diagram of the mechanism of the self-regulating insulin delivery system.

dertaken to separate and analyze the various substituted insulins. The property which may be affected the most by the site and degree of substitution is the bioactivity, and studies have shown that modification can dramatically decrease the potency of insulin. Therefore, high and low potency fractions of glycosylated insulin may be isolated.

Another property of insulin which can be affected by derivatization is its ability to bind to insulin antibodies, which is necessary for detection by radioimmunoassay. It was found that in order to obtain a reliable standard curve in the radioimmunoassay, it was necessary to have a homogeneous preparation of the various glycosylated insulin components.

In addition to its utility in this delivery system, preliminary studies indicate that the gastrointestinal absorption of this derivative may be substantially higher than that of bovine insulin (Haga, 1988). This exemplifies the possible utility of this type of chemical modification in altering the absorption properties of proteins.

Materials and Methods

Materials

All materials were purchased commercially and used without further purification unless otherwise specified. All aqueous solutions were made using double-distilled water. Bovine insulin, *p*-nitrophenyl-α-D-glucopyranoside (NPG), dansyl chloride, succinic anhydride, ammonium formate and α-methyl mannopyranoside were purchased from Sigma Chemical Co. Sodium bicarbonate, sulfuric acid and all organic solvents were at least ACS grade and purchased from Fisher Scientific. All dialysis procedures used Spectropore dialysis tubing (MWCO 1000), and were done against double-distilled water at 4°C.

Synthesis

Synthesis of SAPG-insulin (Fig. 2) was performed using the method developed by Jeong et al. (1984). The first aspect of the synthesis was to reduce the nitrophenyl group on NPG to the corresponding amine functionality (Step A). In order to have a carboxylic group on the sugar conjugate for subsequent amide bond formation with insulin (Step B), *p*-aminophenyl-α-D-glucopyranoside (APG) was reacted with succinic anhydride. Finally, the carboxylic group on succinyl amido phenyl-α-D-glucopyranoside (SAPG) was coupled to the free amines on bovine insulin (Step C) using a mixed anhydride method (Anderson et al., 1967).

During the last conjugation step, there are 3 possible free amines on the native insulin molecule for SAPG to react with: the N-terminal amino groups, A-1 glycine and B-1 phenylalanine, and the amine on B-29 lysine. Thus, the product of this reaction is a heterogeneous mixture of substituted insulins. If all combinations are considered, it is possible to have 3 mono-, 3 di- and one trisubstituted insulin.

Separation

The unreacted insulin was separated from the glycosylated insulin employing the binding affinity of Con A for the glucose or mannose moieties. A sample of 75 mg of the reaction product was dissolved in 100 ml of Tris buffer (0.02 M Tris,

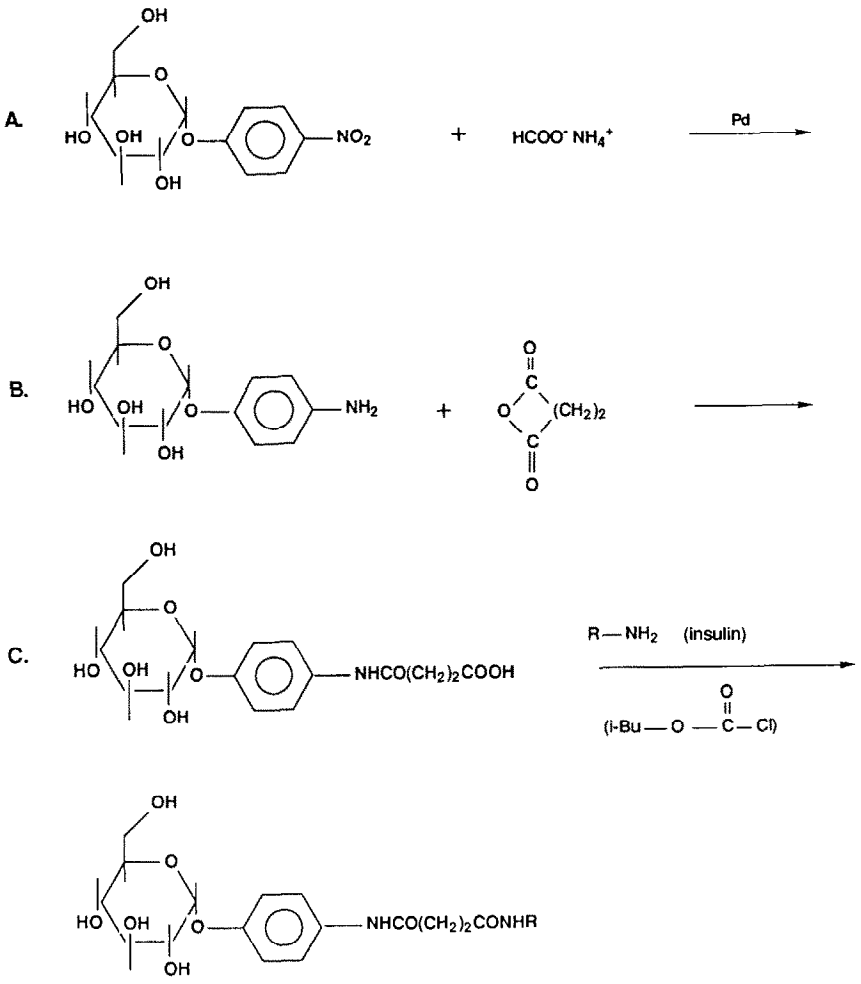

Fig. 2. Synthetic scheme for SAPG-insulin.

pH 7.4) and this mixture was applied to a Con A-Sepharose bead (Pharmacia Fine Chemicals) column, under hydrostatic pressure. The effluent was monitored with an in-line UV monitor (UV-1, Pharmacia Fine Chemicals) at 280 nm and the buffer was allowed to flow through the column until the absorbance of the eluent reached baseline, indicating the removal of the non-glycosylated insulin. The bound glycosylated insulin was displaced from the Con A, by the addition of 0.5 M α-methyl mannopyranoside, collected and extensively dialyzed.

The purified glycosylated insulins were separated into their various substituted derivatives using an automated FPLC (Pharmacia Fine Chemicals), on a Mono Q HR 10/10 anion exchange column (10 mm × 10 cm) at a flow rate of 3.0 ml/min. A 100 mg sample of glycosylated insulin was dissolved in 2.0 ml of starting buffer (5 mM Tris in 8 M urea, pH 8.5), filtered through a 0.45 μm disposable filter, and introduced into the injection loop. The sample was applied to the column and starting buffer was pumped through the column. A preprogrammed gradient was automatically instituted with the final buffer concentration being 280 mM Tris (in 8 M urea at pH 8.5, limiting buffer). The urea (Mallinckrodt, ACS grade) was deionized before use by passing it through an Amberlite MB-3 (Polysciences) column (Offord, 1980). Fractions from each peak

were pooled and extensively dialyzed, lyophilized and stored in the freezer.

End group analysis

End group analysis, performed to determine the amino acid residues on insulin which had been substituted by SAPG, was done by dansylation followed by thin-layer chromatography (TLC). Dansylation was performed according to a procedure outlined by Gray (1967). A 1 mg sample of insulin, SAPG-insulin or fractions of SAPG-insulin, was dissolved in 2.0 ml of 0.5 M $NaHCO_3$/8 M freshly deionized urea and 0.2 ml of 20 mg/ml dansyl chloride in acetone. The sample was incubated at 37 °C until the yellow color of the dansyl chloride had faded (8–12 h), and then dialyzed and lyophilized.

Hydrolysis of the dansylated protein was accomplished using 250 μl of 6 N HCl in a sealed glass ampule at 105 °C for 18 h. The ampule was opened and dried in a vacuum oven, after which TLC was performed on precoated polyamide sheets. The solvents used for two-dimensional TLC were: (a) diethyl ether/methanol/acetic acid (100 : 5 : 1); and (b) ethyl acetate/chloroform/methanol/acetic acid (10 : 10 : 1 : 1). Sample detection was accomplished using a UV lamp.

Sugar group quantitation

The number of sugar groups on the glycosylated insulin was determined using a modified phenol/sulfuric acid technique of Dubois et al. (1956). Vacuum-dried 1 mg samples of glycosylated insulin or fraction of glycosylated insulin were dissolved in 1 ml of double-distilled water and 1 ml of 5% phenol in water. Concentrated H_2SO_4 (5 ml) was added and the tube was allowed to stand for 30 min, after which the tubes were vortexed and the absorbance measured at 490 nm. The amount of sugar was determined using a standard curve for SAPG with the concentration ranging from 0 to 0.1 mg/ml.

Isoelectric focusing

Isoelectric focusing was employed to determine the purity of the separated fractions and was accomplished using premade polyacrylamide gel plates (LKB Instruments) with a pH range of 4.0–6.5. The buffer wicks were saturated with the cathode solution consisting of 0.1 M β-alanine, or the anode solution of 0.1 M glutamic acid in 0.5 M *O*-phosphoric acid provided with the gels. The electrical settings for the power supply (ECPS 3000/150, Pharmacia Fine Chemicals) were 1500 V and 75 mA. The plate was prefocused for 30 min and the samples (2 mg/ml in 8 M urea) were then applied using sample application papers. After 1 h focusing, the application papers were removed and focusing was continued for another hour. At the conclusion of the focusing, the pH at several points on the gel was measured using a surface electrode (Corning). This was followed by refocusing for 10 min to realign any proteins which may have migrated during the pH measurements.

The gels were stained using a method outlined by Steck et al. (1980). The gels were immersed for 1 h in a solution of Coomassie brilliant blue R250 (LKB Instruments) (0.8 g dissolved in a mixture of 420 ml of double-distilled water, 180 ml ethanol and 100 ml of 35% formaldehyde). The gels were destained in a solution of 250 ml ethanol/750 ml double-distilled water/10 ml 35% formaldehyde overnight, with several solution changes in the first 2 h.

Radioimmunossay

Radioimmunoassay for quantitation of insulin was performed using a commercially available kit (Micromedic). However, due to the necessity of accurately quantifying the test insulin, the kit standards were not used. Instead, serial dilutions of the test insulin were used to generate the standard curve. The dilutions were made in Tris buffer (pH 7.4) and linear correlations were obtained only if separated peak fractions were utilized.

Assay for biological activity

This assay was performed using the standard method for determining insulin bioactivity, as described in The British Pharmacopoeia (1968). USP porcine insulin, with an activity of 26 IU/mg, was tested at dose levels of 0.06 IU/0.2 ml and 0.046 IU/0.2 ml in 0.9% saline at pH 2.5. Each glycosylated insulin component was also evaluated at 2 dose levels.

Each insulin dose was tested on a group of 24 mice (50% male and 50% female, 20–23 g). The mice were lightly anesthetized with ether, given a subcutaneous insulin injection and placed in a cage which was partially submerged in a 37°C water bath. The mice were observed for 1.5 h, and those which convulsed or died during this time were removed. The number remaining at the end of this time period were considered survivors. The data was analyzed using probit analysis for a quantal two standard/two sample assay (Yeh, 1981).

TABLE 1

Identification of components of SAPG-insulin

Peak no.	Site of substitution	Degree of substitution (mol SAPG/mol insulin)	Isoelectric point	Biological activity (IU/mg)
I	B-1 Phe	0.78	5.4±0.1	24.7
II	A-1 Gly	0.90	5.4±0.1	22.3
III	A-1 Gly B-1 Phe	–	5.1±0.1	14.8
IV	A-1 Gly B-1 Phe B-29 Lys	–	4.9±0.1	–

Results and Discussion

The relative reactivity of A-1 glycine, B-1 phenylalanine and B-29 lysine amino groups in the insulin molecule, towards most chemical reactions is dependent on many factors, including solvent, pH and the particular reaction mechanism. In general, the B-1 phenylalanine residue is the most reactive, followed by the A-1 glycine, with the B-29 lysine having the lowest reactivity (Blundell et al., 1972; Chan et al., 1981). Structurally, both the A-1 glycine and the B-1 phenylalanine occupy surface pockets within the intact insulin hexamer, making them fairly accessible. However, upon dissociation to the dimer the B-1 group becomes more readily accessible. This dissociation may be promoted by DMF (the solvent used in the coupling reaction) which may disrupt hydrogen bonding in the native insulin structure. Therefore, it was expected that the relative yield of the B-1 phenylalanine monosubstituted derivative would be larger than that of the A-1 glycine monosubstituted conjugate.

The FPLC chromatogram of SAPG-insulin before separation on the Con A column was complex and contained many unresolved peaks. In addition, bovine insulin eluted close to the same position as one of the SAPG-insulin peaks (at an elution volume of 125 ml). Therefore, affinity chromatography was used as an additional purification step.

Fig. 3 shows the FPLC chromatographic profile of SAPG-insulin after the removal of insulin and other side products on a Con A affinity column. This chromatogram is much less complex, and complete resolution between the 4 peaks (I, II, III

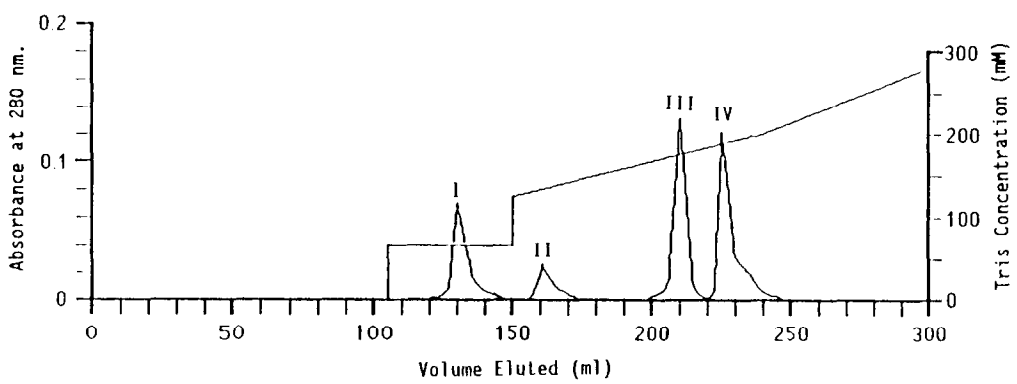

Fig. 3. FPLC chromatogram of SAPG-insulin on an anion exchange column after purification by Con A affinity chromatography.

TABLE 2

Biological activity of SAPG-insulin

Insulin type	Dose (μg)	Percent convulsed	Calculated potency (IU/mg)	Fiducial limits ($P = 0.95$)
SAPG	1.84	33.3	24.7	21.5–27.7
peak I	2.40	79.2		
SAPG	2.08	48.8	22.3	18.9–25.7
peak II	2.72	66.7		
SAPG	2.40	4.2	14.8	11.0–17.0
peak III	3.12	33.3		
SAPG	2.40	54.2	21.6	19.4–24.7
unseparated	3.12	95.8		
bovine	1.84	54.2	25.0	19.9–29.8
	2.40	70.8		

and IV) was obtained. The 4 major insulin peaks were isolated and characterized, these results are summarized in Table 1. Detailed biological activity data and statistical analysis are given in Table 2.

The major end groups found by the dansylation method for peak I of SAPG-insulin were the dansylated ϵ-lysine, glycine and tyrosine residues (see Fig. 4A). For reference, the TLC profile of dansylated bovine insulin is shown in Fig. 5. The dansyl tyrosine was seen on all the other samples and is an expected byproduct. Therefore, peak I was identified as B-1 phenylalanine monosubstituted SAPG-insulin. This is consistent with predictions that the B-1 phenylalanine α-amino group would be the most reactive of the 3 amines on insulin (Blundell et al., 1972). In addition, sugar group quantitation yielded an average value ($n = 3$) of 0.78 mol SAPG/mol insulin which is reasonably close to the expected value of 1.0 for a monosubstituted insulin. The low experimentally determined degree of substitution may be due to the quenching of the SAPG on insulin during spectrophotometric measurement. The standard used was free SAPG which has considerable more mobility because it is not covalently bound to a large molecule (i.e. insulin).

The pI of this fraction was slightly lower than native insulin, 5.6 ± 0.1, because substitution of

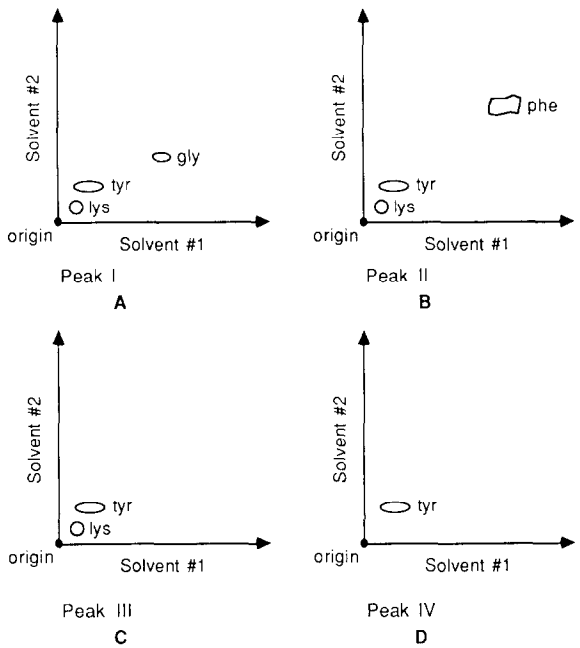

Fig. 4. Two-dimensional TLC profiles of dansylated hydrolysates of each peak fraction of SAPG-insulin.

the free amines on insulin results in a more negatively charged molecule, which requires a higher H^+ concentration (lower pH) to attain neutrality. The biological assay showed that the activity of the peak I fraction, 24.7 IU/mg, was comparable to that of native insulin. A result which has been documented by other investigators with B-1 phenylalanine monosubstituted insulins (Blundell et al., 1972; Lindsay and Shall, 1971).

The peak II fraction of insulin was identified, by end group analysis, (see Fig. 4B) as A-1 glycine

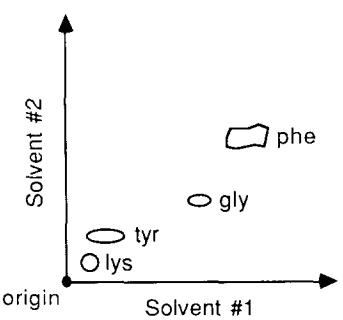

Fig. 5. Two-dimensional TLC profiles of dansylated hydrolysates of bovine insulin.

monosubstituted insulin. Also, sugar group quantitation yielded a value of 0.90 mol SAPG/mol insulin, which is consistent with the finding that this component was a monosubstituted insulin. This fraction had a p*I* of 5.0 ± 0.1, with a slight decrease in biological activity (22.3 IU/mg). A decrease in activity upon substitution of the A-1 glycine residue has been documented by other researchers (Dodson et al., 1979; Ellis et al., 1978; Gliemann and Gammeltoft, 1974). Although in many cases, modification of the amino residue of A-1 glycine by moieties larger than acetate causes a severe loss in activity, this is not always the case. The diminished biological activity is thought to occur due to the direct involvement of the A-1 glycine in insulin receptor binding (Chan et al., 1981). It is hypothesized that modification of the A-1 glycine may distort the hydrogen bonding between the glycine residue and adjacent atoms, thereby causing an alteration in the tertiary structure necessary for full biological expression. Since a decrease in the activity of this derivative was not observed, modification of insulin by SAPG may not significantly disrupt the tertiary structure of the protein.

Peak III was identified as A-1 glycine, B-1 phenylalanine disubstituted SAPG-insulin based on end group analysis (Fig. 4C). The p*I* of this conjugate was substantially lower (4.6 ± 0.1) than unsubstituted insulin (5.6 ± 0.1), as would be expected upon disubstitution of the amines. The biological activity of this peak was also lower than any of the other fractions, (14.7 IU/mg). This reduction in potency with both A-1 glycine and B-1 phenylalanine substituted moieties is somewhat surprising, since the A-1 glycine monosubstituted derivative was biologically active. However, it has been found that insulin derivatized with alkyl groups at the A-1 and B-1 positions has almost no biological activity (Gliemann and Gammeltoft, 1974). In addition, insulin with a cross-link between the A-1 and B-1 residues has been shown to have reduced biological potency and receptor binding. The cross-link was believed to prevent a conformational change necessary for full expression of insulin's biological activity (Dodson et al., 1979). Although this disubstituted insulin may not be covalently crosslinked it is possible that there is some interaction between the SAPG substituents at the B-1 and A-1 positions which leads to its behavior as a crosslinked derivative.

The last peak (IV) was identified as trisubstituted SAPG-insulin, as evidenced by dansyl tyrosine being the only amino acid present after the end group analysis (Fig. 4D). This sample was isolated in such small quantities after the purification and separation steps that it made other testing difficult, and further experiments with this fraction were not performed. However, it would be expected that the biological activity of this sample would be low, and comparable to that of the peak III fraction, due to the effect of the A-1 glycine and B-1 phenylalanine substitutions.

The biological activity of unseparated SAPG-

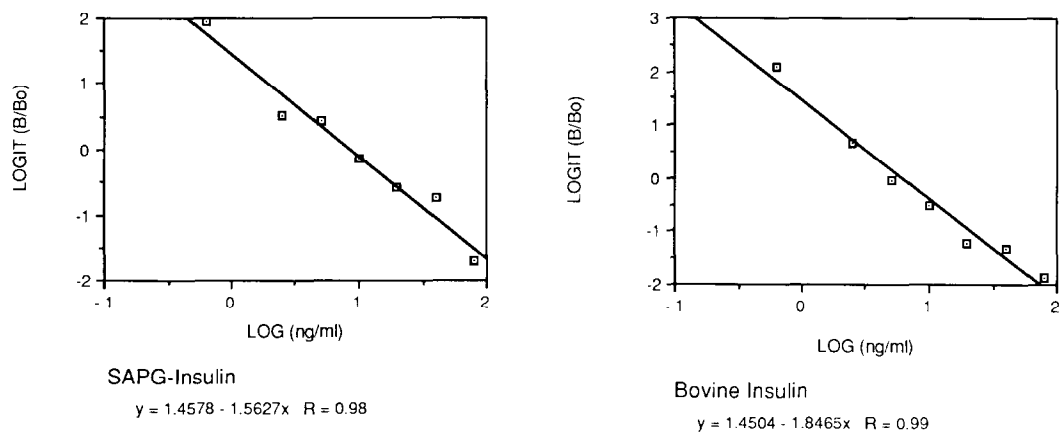

Fig. 6. RIA standard curves for bovine and SAPG-insulin.

insulin was found to be 21.6 IU/mg (Table 2), reflecting the effect of the lower potency of the peak III and IV fractions. Therefore, from the standpoint of bioactivity, it is advantageous to use the separated peak I or II fractions of SAPG-insulin in this delivery system. The yield of the peak I fraction is greater than the peak II fraction, therefore the final prototype will utilize peak I SAPG-insulin.

RIA cross-reactivity of the peak I fraction of SAPG-insulin was good and was quite similar to that of insulin. Standard curves of percent bound to insulin antibody versus insulin concentration are shown in Fig. 6. Graphs for bovine insulin and SAPG-insulin (peak I) show linearity over the concentration range of 0–100 ng/ml. However, RIA on unseparated glycosylated insulin yielded nonlinear plots, indicative of the different affinities of the different components for the insulin antibody (Blundell et al., 1972). These results indicate that the glycosylated insulin is immunoreactive and RIA is a reasonable method to determine glycosylated insulin concentration, both in vitro and in vivo.

While the ability to detect glycosylated insulin via RIA indicated that it is immunoreactive, this does not necessarily mean that it is antigenic in vivo. The ability of the insulin derivative to bind to the antibody in vitro should not be confused with its capability to stimulate antibody production in vivo.

Conclusions

These studies have shown the heterogenous nature of the unseparated glycosylated insulin SAPG-insulin. However, the two chromatographic procedures, Con A affinity chromatography and FPLC have provided suitable resolution between the 4 SAPG-insulin components. The components were the monosubstituted B-1 phenylalanine insulin conjugate, the A-1 glycine monosubstituted complex, disubstituted insulin derivatized at both the A-1 and B-1 amines and the A-1, B-1, B-29 trisubstituted SAPG-insulin.

Characterization of the various fractions resulted in the identification of each peak and the biological assay demonstrated that the B-1 phenylalanine and A-1 glycine monosubstituted component of SAPG-insulin were the most biologically potent. Since the B-1 phenylalanine monosubstituted SAPG-insulin constituted the largest reaction product, it is an ideal candidate for use in the self-regulating insulin delivery system. In addition, the concept of enhancing gastrointestinal absorption by glycosylation may prove useful in the development of orally administered peptides and proteins.

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