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PRODUCTION SCALE PURIFICATION OF BIOSYNTHETIC HUMAN INSULIN BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A process based on reversed-phase high-performance liquid chromatography (RP-HPLC) has been developed for the purification of biosynthetic human insulin (BHI). The RP-HPLC procedure has been successfully integrated into the multimodal chromatographic production process used to purify kilogram quantities of BHI. Axial compression column technology was used in the scale-up process. The RP-HPLC procedure yields an insulin product having high chemical purity and full biological activity.

INTRODUCTION

Recombinant DNA technology has provided the means for preparing virtually unlimited quantities of therapeutically important peptide and protein products. A recent example of the successful use of this technology is the production of the peptide hormone human insulin, using *Escherichia coli*[1–7]. While genetic engineering and associated fermentation technology provides the initial protein product, the protein must be extensively purified and processed before it can be formulated into a final dosage form. A purification process based on multimodal chromatography, which exploits differences in molecular charge, size, and hydrophobicity, is typically used to isolate the human insulin[6–8]. Such a purification scheme must be capable of efficiently isolating the insulin product from the very complex fermentation matrix. In addition, the insulin purification process should be capable of reducing the levels of insulin-like components, that is, those components resulting from minor modifications of the insulin molecule itself. The reduction of these components can be particularly challenging, since in many cases the insulin-like substances differ in the modification of only a single amino acid residue.

We have investigated the use of reversed-phase high-performance liquid chro-

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matography (RP-HPLC) as an integral step in the multimodal chromatographic purification of biosynthetic human insulin (BHI). RP-HPLC is a logical choice, since its selectivity and high resolving capabilities have been previously exploited in the field of insulin analysis. For example, insulin species which differ by a single amino acid residue have been successfully resolved using a wide variety of stationary and mobile phases[1,9–17]. It has been demonstrated that the technique can be very useful for the separation and quantitation of a variety of insulin-like components[11–20]. Modified forms, such as desamido, carbamoyl, formyl, and insulin polymers, have been successfully separated from the parent insulin molecule. RP-HPLC has also proven to be an invaluable tool for monitoring the quality and quantity of insulin throughout the isolation and purification process[20]. In addition, important chemical intermediates such as A- and B-chain S-sulfonates, and proinsulin and its S-sulfonate have been characterized by RP-HPLC[16,20,21].

The isolation and purification of insulin and insulin derivatives has also been investigated by preparative RP-HPLC. In these studies, analytical-scale columns were employed to isolate micro- to milligram quantities of the desired product. The purification of iodinated insulins, commonly used as tracers in radioimmunoassays and receptor assays, has been reported by a number of groups[16,22,23]. In these studies, [^{125}I]insulin isomers were successfully separated and isolated in a single run. Genetic variants of insulin have been detected and isolated from blood samples of diabetics by RP-HPLC[24]. Proinsulin, the biological precursor to insulin, has also been purified by this technique. The RP-HPLC procedure was used to purify the bovine proinsulin present in the high-molecular-weight fraction obtained from size-exclusion chromatography of insulin[25]. In another study, a variant of bovine proinsulin was isolated by reversed-phase chromatography[26]. In a recent report, the levels of proinsulin associated with preparations of bovine and porcine insulins were reduced by RP-HPLC displacement chromatography[27]. In this study, up to 500 mg of insulin were purified on analytical columns of Nucleosil C_8, using centrimide as a displacing agent.

In the current paper, we will discuss the use of preparative RP-HPLC for purifying production scale quantities of BHI. We will examine the critical chromatographic operating parameters, the scale-up of the process, and the practical aspects of integrating this procedure into a multimodal purification scheme.

EXPERIMENTAL

Equipment

Laboratory scale purification studies were conducted with either a Varian (Walnut Creek, CA, U.S.A.) 5000 gradient HPLC system equipped with a Du Pont (Wilmington, DE U.S.A.) variable-wavelength detector set at 280 nm, or a Pharmacia (Piscataway, NJ, U.S.A.) FPLC system composed of an LCC 500 controller, two P-500 pumps, and a UV-2 detector system with lamp and filters for detection at 280 nm. The column effluent from both systems was fractionated using a Pharmacia Frac-100 fraction collector.

A Rainin (Woburn, MA, U.S.A.) Rabbit gradient system, composed of two model HP pumps fitted with 100-ml preparative heads and controlled with an Apple 2E computer system, was used for studies with the 50 × 5.0 cm I.D. column.

Pilot-plant and production-scale purification studies were conducted on LC 150 VE, LC 300, or LC 450 axial compression columns, available from Prochrom (Nancy, France). All units were equipped with dual-head high-pressure solvent delivery pumps and automation units for producing gradients. The columns were fitted with 2-μm frits (Mott Metallurgical, Farmington, CT, U.S.A.).

Materials

The stationary phases evaluated for the purification studies were obtained directly from the manufacturers as prepacked analytical columns or as bulk packings. The 10-μm Zorbax Process Grade C_8, used for the pilot-plant and production-scale purification studies was obtained from DuPont.

The partially purified human insulin zinc crystals used in the purification studies were prepared at Eli Lilly and Company (Indianapolis, IN, U.S.A.). The human insulin derivatives were prepared in the Lilly Research Laboratories Human insulin reference standard lots P99843 and RS0026 were used for the HPLC and for the biopotency assays.

All reagents used in these studies were of reagent grade quality or better and were obtained from a variety of suppliers.

Procedures

Packing of fixed-bed columns. Columns for the initial laboratory studies were packed with the desired stationary phase, using a high-pressure, non-balanced slurry-packing technique[28]. Columns and packing reservoir (HT Chemical, St. Louis, MO, U.S.A.) were fitted to a Model DSTV 72C Haskel pump (Burbank, CA, U.S.A.). The desired stationary phase was slurried in acetonitrile at a ratio of 1 g of packing per 2 ml of solvent. Morpholine at a concentration of 0.02% was added to prevent agglomeration of the particles. The slurry was sonicated for approximately 10 min and loaded into the packing reservoir; acetonitrile was then pumped through the system at a flow-rate high enough to generate 3000 p.s.i. (5000 p.s.i. for columns having internal diameters $\leq$ 1.0 cm).

Packing of axial compression columns. The stationary phase of choice was slurried with acetonitrile in a ratio of 1 kg of packing per 2 l of acetonitrile. Typically, 5-kg, 25-kg, and 50-kg quantities of packing were slurried for the 15-cm, 30-cm and 45-cm I.D. axial compression columns, respectively. The hydraulic piston of the column was lowered to a level to accommodate the volume of the slurry. The slurry was transferred to the column by means of an air diaphragm pump, the top flange was replaced, and the piston was raised, displacing the acetonitrile. The packed column was maintained at a pressure of 750 p.s.i. by the piston during column operation.

Evaluation of packed columns. The efficiency of the packed columns was evaluated with methyl benzoate as a test probe. The columns were equilibrated with 60% aqueous acetonitrile at a flow-rate of 3 cm/min. Flow was maintained in the direction of packing. A solution of 90% aqueous acetonitrile containing 130 μg of uracil and 30 μl of methyl benzoate per ml was introduced into the column with a loop injector (5–15 ml for the preparative columns and 20 μl for analytical columns). The chromatogram was recorded and the plate count was determined, using the half height procedure[28]. Plate counts of 30 000–40 000 plates/m were obtained for the fixed-bed columns, and 45 000–55 000 plates/m were common for the axial compression columns.

Purification studies. The column to be tested was equilibrated in 0.25 *M* acetic acid–acetonitrile (90:10). The BHI zinc insulin crystals to be purified were dissolved in the column equilibration buffer at a concentration of 15 mg/ml. The insulin solution was loaded onto the column at a flow-rate of 1.5 column volumes/h. (Note that unless otherwise stated, the term column volume (CV) refers to the volume of the *empty* column). Columns were typically loaded with 12–15 mg of insulin/ml of CV. After loading, the column was washed with 1 CV of the initial equilibration buffer and the gradient of choice was run, using 0.25 *M* acetic acid as eluent A and 60% aqueous acetonitrile as eluent B. During elution, 0.1-CV fractions were collected. The fractions were assayed with the analytical HPLC systems described below, and the desired fractions were pooled. During pilot-plant and production-scale studies, insulin was recovered from the mainstream pool by means of a variation of the zinc crystallization procedure described previously[29]. Briefly, the concentration of insulin is adjusted to 2 g/l with 0.25 *M* acetic acid, the pH is adjusted to 5.9 with ammonium hydroxide, and a 1.8 molar excess of zinc chloride over BHI is added. The zinc insulin crystals formed are isolated by filtration.

Analytical characterization of BHI

Analytical HPLC. Several analytical RP-HPLC systems were used to evaluate the quality and quantity of BHI throughout the purification process. Mobile phases containing the ion-pair reagents pentanesulfonic acid (PSA) or octanesulfonic acid (OSA) were used to characterize the BHI at the RP-HPLC purification step[30]. The PSA mobile phase was composed of 20 m*M* PSA in acetonitrile–Milli Q water (31:69) (pH 2.1, adjusted with phosphoric acid). The OSA mobile phase consisted of 20 m*M* OSA and 33m*M* phosphoric acid, in Milli Q water–acetonitrile (57:43) (pH 2.0, adjusted with phosphoric acid). Chromatography was conducted on a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1090 liquid chromatograph fitted with two Zorbax C_8 (Du Pont), 150-Å columns (15 cm × 0.45 cm I.D., connected in series), thermostatted to 30°C and operated at a flow-rate of 0.8 ml/min. An alternative column system employed two Zorbax C_8 Reliance cartridge columns (connected in series), thermostatted to 35°C and operated at a flow-rate of 0.8 ml/min. The column effluent for all of the systems was monitored at 214 nm, and 20-µl injection volumes were typical. The detector output was analyzed by a chromatographic data-handling system, based on a Hewlett-Packard Series 1000 computer. The insulin concentration was determined by comparing the insulin peak area response of the sample to that of a standard, while the insulin purity was determined by comparing the area of the insulin peak to the total peak area in the chromatogram, disregarding the solvent front.

The RP-HPLC gradient system used to characterize the final BHI crystals has been reported previously[12].

E. coli polypeptides (ECPs). The radioimmunoassay system for detecting ECPs has been described previously[31]. The limit of detection is approximately 4 ppm.

Determination of bonded phase in BHI crystals. Final BHI crystals were checked for the presence of reversed-phase breakdown products (siloxanes) using a gas chromatographic assay procedure. Insulin (1 g) was extracted with 5 ml of dichloromethane for 30 min. The insulin was filtered, and 1 µl of the dichloromethane extract was injected into a Hewlett-Packard Model 5890 gas chromatograph, fitted with a

15 m × 0.53 mm I.D. Megabore DB-5 column (J. and W. Scientific, Folsom, CA, U.S.A.). The injection temperature was set to 200°C, and the temperature of the flame-ionization detector was set to 250°C. The column temperature was maintained at 45°C for 2 min, followed by a temperature program of 12°C/min for 12 min. The final column temperature of 200°C was held for an additional 8 min. Potential bonded phase breakdown products were obtained from Petrarch Systems (Bristol, PA, U.S.A.) and were dissolved in dichloromethane. These compounds included: *n*-octyldimethylsiloxane; 1,3-bis(*n*-octyl)-tetramethyldisiloxane; *n*-octylpentamethyldisiloxane; and hexamethylcyclotrisiloxane. The limit of detection for these compounds in final insulin crystals is approximately 10 ppm.

Biopotency assay of final zinc crystals. The hypoglycemic potency of final BHI zinc crystal lots was determined by the USP rabbit assay.

Limulus amebocyte lysate (LAL) assay. The assay has been described previously[32].

RESULTS AND DISCUSSION

In designing and implementing a reversed-phase purification step for BHI, a number of practical goals and objectives were considered. First, the procedure must be capable of producing a high-quality insulin product in good yields. Purities of >97.5% (RP-HPLC) with mainstream yields of ≥75% served as minimum acceptable goals. Second, the process should be capable of generating high-purity insulin derived from different feed sources. The level of insulin-like components present in these feeds could range from 6 to 20%, depending upon the nature of the feed source. Third, the operational parameters must be compatible with physicochemical properties of the insulin molecule. The conditions must provide for good solubility, without subjecting the insulin either to extremes in pH or to conditions that would cause irreversible denaturation. Fourth, the process should complement the other steps in process. That is, it should enhance the multimodal purification scheme as well as produce a product that can be easily taken into the next phase of purification. Fifth, the process must be capable of being implemented in a production environment. The process, along with any necessary ancillary equipment, should be readily scalable. It must also be capable of operating in a very reproducible manner. Finally, the procedure must be economically viable. This necessitates good process yields and good packing lifetime. Additionally, the procedure should be able to replace conventional chromatography steps and/or purify material which otherwise could not be purified by conventional procedures.

Production of BHI

The production of biosynthetic human insulin (BHI) can be accomplished by either the A- and B-chain[1,5–7] or the proinsulin[2,5–7] processes described in Fig. 1. In the chain process, the A- and B-chains which comprise the insulin molecule, are separately fermented, isolated, and purified in the form of stable S-sulfonate derivatives. The chains are then combined to form the insulin molecule which is subjected to conventional ion-exchange and size-exclusion chromatography. During the course of the chromatographic purification, fractions are generated that contain appreciable quantities of insulin but which are not pure enough to be included in the mainstream.

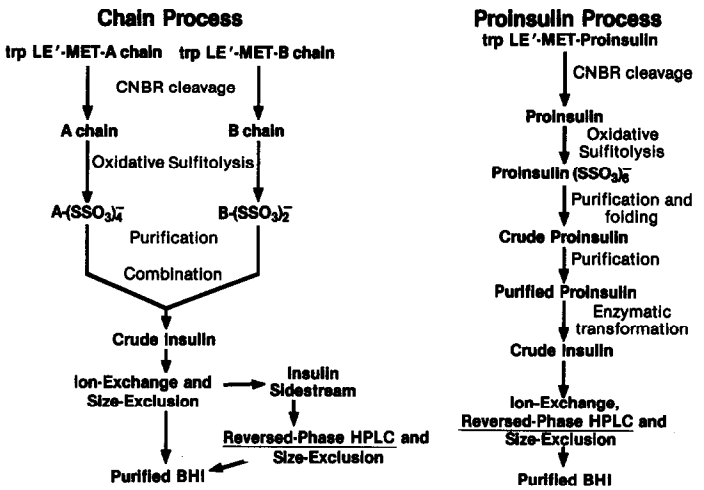

Fig. 1. Production of BHI by chain and proinsulin processes.

In particular, the levels of insulin-like components are enriched in these sidestream fractions. The quantity of purified insulin obtained by recycling the sidestreams through the original process is not great enough to make recycling feasible. These insulin sidestreams are therefore excellent candidates for the high resolving capability of RP-HPLC.

The proinsulin route, which is the current method of choice for producing human insulin, includes RP-HPLC as a routine step in the overall purification scheme. The proinsulin route has proven to be a more efficient method for preparing BHI, since only a single fermentation isolation scheme is necessary. In this procedure, the S-sulfonate derivative of proinsulin is isolated and chromatographically purified. The S-sulfonate is then folded to form proinsulin, which is further purified before being enzymatically transformed to BHI. The resulting BHI is purified by ion-exchange chromatography and is crystallized. The redissolved crystals serve as the charge material for the reversed-phase procedure. Following reversed-phase purification, the insulin is purified by size-exclusion chromatography and crystallization.

In both of the synthetic routes, placement of the reversed-phase step within the overall purification scheme was based on a number of practical and procedural considerations. First, it is the insulin molecule itself which is to be purified by HPLC. This requires that the reversed-phase step be placed after either the combination of the chains or the enzymatic conversion of proinsulin to insulin. Second, by inserting the step late in the overall process, we can take advantage of the fact that the majority of the *E. coli*-derived impurities, which arise from the bacterial host system, have been removed by the preceding steps. It is necessary to minimize these impurities since they reduce the effective lifetime of the stationary phase by physically and/or chemically fouling the packing due to low solubility or irreversible binding[20]. This is critical, since the silica-based packings have pH limitations which preclude the use of pH extremes for clean-up and regeneration. Third, the HPLC procedure is designed to be a "polishing" step. It is intended to reduce the level of insulin-like components which

are generated early in the isolation purification scheme and which are difficult to remove by conventional chromatographic procedures. In addition, the reversed-phase step complements the ion-exchange step that precedes it and the size-exclusion step which follows. Insulin is therefore subjected to three different modes of purification. Finally, because every purification process incurs progressive yield losses, a smaller amount of material will need to be processed through later steps than through the initial steps. For the reversed-phase procedure, this translates into fewer runs and smaller amounts of packing material.

Chromatographic parameters

A variety of chromatographic parameters was evaluated and optimized on small columns (15 × 0.94 cm I.D.), using laboratory equipment, before scale-up of the process was undertaken. A brief summary of the critical operational parameters follows.

Stationary phase. A number of stationary phases (C_4, C_8, C_{18}) produced by a variety of manufacturers (Du Pont, Vydac, Amicon, Synchrom) were initially evaluated for their ability to purify insulin. In general, ≤ 12-μm silica particles, derivatized with C_8 or C_{18}, yielded the best results. Pore sizes of 120 to 150 Å were ideal for this application, since they were large enough to permit efficient mass transfer of the insulin in and out of the pores, yet small enough to provide sufficient surface area for this preparative application. In addition, the large-pore silicas (≥ 300 Å) proved to be more fragile than the smaller-pore materials. Greater care was required in packing these particles, since they were more friable and, hence, gave rise to "fines", which caused excessive pressures. While there were a number of commercial packings which possessed the desired chemical and physical properties for this application, the need for large quantities of packing material limited our available options. The 10-μm Zorbax Process Grade C_8 was available in large supplies and met our chromatographic criteria. By working closely with the manufacturer and designing a laboratory scale preparative evaluation system, we were assured of lot to lot reproducibility. More recently, other manufacturers have begun to produce small-particle, reversed-phase stationary phases in large bulk quantities, and these packings could prove suitable for similar production scale applications.

Mobile-phase pH and buffer. Analytical studies with a variety of reversed-phase systems, have shown that an acidic mobile phase can provide excellent resolution of insulin from structurally similar insulin-like components[10–19]. Minor modifications in the insulin molecule resulting in monodesamido (A-21) formation, or derivatization of amines via carbamoylation or formylation, result in insulin derivatives which have significantly increased retention[12,13]. Derivatives of this nature are typical of the kind of insulin-like components that are found in the charge stream going into reversed-phase purification. Fig. 2 shows the elution order of these common derivatives under both acid and alkaline mobile-phase pH conditions. Note that in the mildly alkaline pH elution scheme, the derivatives are eluted on either side of the parent insulin peak. By contrast, under acidic elution conditions, all of the derivatives are eluted after the insulin peak. The ideal elution scheme should uniformly concentrate and elute the insulin-like components on one side of the insulin peak. Additionally, the yield of purified insulin is expected to be maximized if insulin is eluted *ahead* of the components to be removed[33]. Thus, the acidic mobile phase is the better choice here.

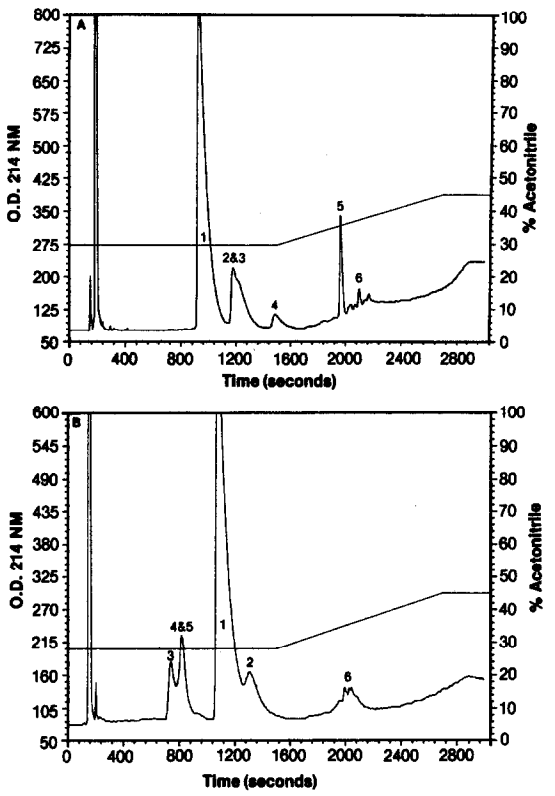

Fig. 2. Elution of BHI and BHI derivatives in RP-HPLC with (A) acid and (B) alkaline mobile phases. Column, Zorbax C_8, 150 Å (25 × 0.45 cm I.D.), thermostatted to 35°C; flow-rate, 1.0 ml/min; sample load, 7.5 μg BHI and 1.3 μg of BHI derivatives. Solutes: 1 = BHI, 2 = desamido A-21 BHI, 3 = N-carbamoyl-Gly BHI, 4 = N-formyl-Gly BHI, 5 = N-carbamoyl-Phe BHI, 6 = BHI dimers. Mobile phases: (A) eluent A = 0.1 *M* phosphate (pH 2.1), eluent B = eluent A–acetonitrile (1:1); (B) eluent A = 0.1 *M* phosphate (pH 7.3), eluent B = eluent A–acetonitrile (1:1).

An ideal pH for insulin purification is in the region of 3.0–4.0, since this pH range is far enough below the isoelectric pH of 5.4 to provide for good insulin solubility. Under acidic conditions, insulin can deamidate to generate monodesamido (A-21) insulin[34]. However, because the RP-HPLC procedure can be conducted within a matter of hours, and the resulting insulin can be removed from the acidic environment via zinc crystallization, deamidation is not a significant problem. Additionally, the mildly acidic conditions will minimize the formation of monodesamido (B-3) insulin, which can be generated at pH 7 or slightly above[34,35]. The mildly acidic mobile phase is also compatible with the pH limitations of the stationary phase (2.0–8.0), as it is low enough to prevent dissolution of the silica support and high enough to minimize hydrolysis of the alkyl chain[36]. Stationary phase stability is critical in both preventing contamination of the insulin with these potential breakdown products and insuring a reasonable column lifetime.

The type of acidic buffer used in the mobile phase is also important in this application. Insulin can undergo self-association and aggregate. This, in turn, can

lead to gel formation and precipitation[37,38]. Gel formation is accelerated when insulin is present in high concentrations at low pH and high electrolyte concentrations. The presence of organic solvents can aggravate this problem. Thus, the mobile phase buffer should be capable of maintaining a stable pH without the addition of salts. The buffer of choice must also be compatible with downstream processing. In this instance, zinc crystallization of the reversed-phase mainstream was the method of choice, since it provides an efficient and convenient means of concentrating and holding the insulin before further processing.

The buffers evaluated for this application included sodium phosphate, formic, acetic, and propionic acids. Acetic acid, at a concentration of 0.25 *M* in eluent A, meets all of the operational criteria. It is compatible with the chromatography and provides good insulin solubility. It buffers in the vicinity of pH 3 without the need for other salts; and, insulin can be readily crystallized from the acetic acid containing mobile phase by the addition of zinc chloride. Acetic acid-containing mobile phases have also been widely used for the size-exclusion chromatography of insulins[13,18,26,27,29,39,40]. In addition to allowing insulin concentrations to exceed 50 g/l, it serves to dissociate the insulin hexamer into a monomer, which is more suitable for chromatography.

Organic modifier. The organic modifier used to elute insulin from the stationary phase should provide both the desired chromatographic selectivity and good insulin solubility. It should have a low viscosity in order to minimize the back pressure in the system, and should be readily available in large quantities. A variety of solvents, including: ethanol and isopropanol, acetonitrile and acetone were considered. Ethanol and isopropanol gave poor resolution of the insulin-like components from insulin and resulted in mainstream yields of only 50–60%. In addition, the solubility of insulin was low in the isopropanol-containing mobile phase and isopropanol gave rise to back pressures *ca.* 1.2–1.5 times greater than for ethanol. Acetone resulted in very poor mainstream yields ($< 50\%$), due to poor insulin solubility. Acetone furthermore precludes monitoring of the process stream at 280 nm. Acetonitrile was the best solvent for this process, as it provides good chromatographic selectivity, insulin solubility, and results in lower operating back pressures than isopropanol. The use of acetonitrile as an organic modifier for analytical and semi-preparative insulin separations has been widely documented[1,2,9–26]. Preparative studies with acetonitrile demonstrated that mainstream yields on the order of 75–85% could be readily obtained (see *Insulin purification*). Acetonitrile can be obtained in bulk quantities from a variety of suppliers and, if desired, can be recovered by distillation. In addition, insulin can be readily recovered from the mobile phase by the addition of zinc chloride and acetic acid, since the presence of acetonitrile at levels $\leq 10\%$ does not interfere with the formation of zinc insulin crystals.

Loading and elution scheme. A chromatographic procedure in which insulin is initially loaded onto the stationary phase in a water-rich mobile phase, followed by gradient elution with acetonitrile, was determined to be ideal for this application. This type of load and elution scheme allows the insulin to be concentrated on the column and then eluted in a volume of solution equivalent to one column volume (empty) or less. By contrast, insulin loaded and eluted under isocratic conditions was typically eluted in 2 or more CV. The small volume of solution resulting from the gradient elution conditions, makes handling and processing of the insulin much easier.

The capacity of the stationary phase for insulin was determined to be approximately 85 mg of BHI/ml of packing. This was determined by loading the insulin charge solution onto a 15 × 0.94 cm I.D. column (10.4 ml) at a flow-rate of 1.5 CV/h (*ca.* 0.3 ml/min) and monitoring the column effluent for insulin concentrations ≥ 0.1 mg/ml. The capacity was defined as being the total quantity of insulin on column at the point of breakthrough, divided by the volume of packing (10.4 ml). For purification studies, loadings comparable to approximately 18% of the capacity of the packed column were used.

Insulin elution was accomplished with a linear gradient, typically ranging from 15 to 30% acetonitrile. Gradient elution was found to be superior to stepwise elution. While stepwise gradients are easier to generate and are therefore preferred for large-scale applications, it was impossible to find a concentration of acetonitrile that would provide a high quality product ($\geq 97\%$) with good recovery ($\geq 75\%$) in a manageable volume (≤ 1 CV). The overload condition under which this procedure is operating gives rise to a non-linear adsorption isotherm. Under this condition, a continuous gradient elution scheme was necessary to produce the desired results of high purity with good mainstream yield. The gradient volume was found to have a significant effect on product quality, mainstream recovery, and volume. A gradient of 15 to 30% acetonitrile running over 8 CV yielded the best results. This represents a gradient slope of approximately 2% acetonitrile/CV.

Regeneration. After elution of the insulin, it is necessary to regenerate the column in order to remove any strongly retained components. The regeneration process reduces the potential for column fouling and helps to increase column lifetime. Effective elution of these strongly retained components is accomplished by increasing the organic modifier concentration to 60%, and by increasing the pH of mobile phase to 7.4 by using a 50 m*M* ammonium phosphate buffer. It is important to note that both the increase in organic modifier concentration and the change in mobile phase pH are necessary to regenerate the column effectively. Detailed regeneration studies indicate that the alkaline buffer is necessary in order to elute a small portion of insulin and other components which are retained by secondary silanol interactions. Elution is further enhanced by increasing the organic modifier concentration. With this regeneration procedure, quantitative recovery ($\geq 97\%$) of insulin and total protein can be obtained.

Insulin purification. With the parameters discussed above, a series of laboratory-scale purification studies were conducted before scale-up was pursued. Fig. 3 shows a typical elution profile for the preparative purification of BHI. The charge for this study was derived from the proinsulin process. The loading and elution conditions are outlined in the legend and are similar to those discussed above. The overload condition does not permit resolution of the various components as judged from the UV profile. However, when the collected fractions are subjected to analytical RP-HPLC, separation of insulin-like components from the insulin becomes very clear. Fractions having a purity of $>96\%$ were included in the mainstream. Fig. 4 shows the analytical chromatograms for the column charge and purified mainstream. In addition, a profile for the sidestream is included. It is interesting to note that while the insulin-like components are eluted after the main peak in the preparative system (at higher acetonitrile concentrations and during the column regeneration), these components eluted on either side of the insulin peak in the analytical system. The ion-pairing reagent,

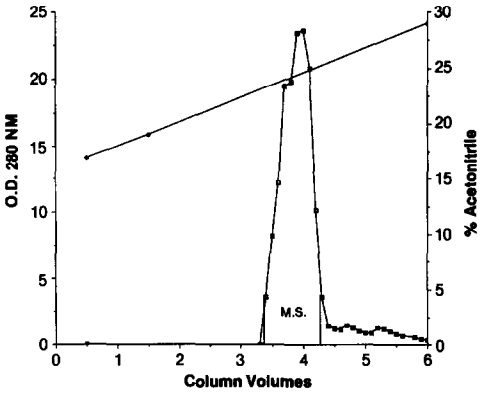

Fig. 3. Preparative chromatogram for BHI. Column, 10-μm Zorbax Process Grade C_8 (15 × 0.94 cm I.D.); load, 153 mg of BHI derived from proinsulin process; gradient, 17 to 29% acetonitrile in 0.25 *M* acetic acid, in 6 CV; flow-rate, 0.3 ml/min. Fractions 3.3 to 4.3 column volumes were pooled (mainstream). Fractions 3.2, 4.4–5.4, and the protein eluted in column regeneration (not shown) were combined (sidestream).

present in the mobile phase of the analytical system, affects the elution order of these components relative to the main insulin peak[30]. The purity of the mainstream in this case is 98.7% compared to *ca.* 91.5% for the starting material. The average mainstream insulin yield for five such preparative experiments was 82%, an additional 15% being present in the sidestream and regeneration solutions, giving an overall insulin yield of 97%.

Scale-up of the purification process

Having optimized the parameters for the insulin purification process on small laboratory columns, we increased column sizes in a series of scale-up experiments. Table I summarizes the column sizes used in these studies. As we progressed through

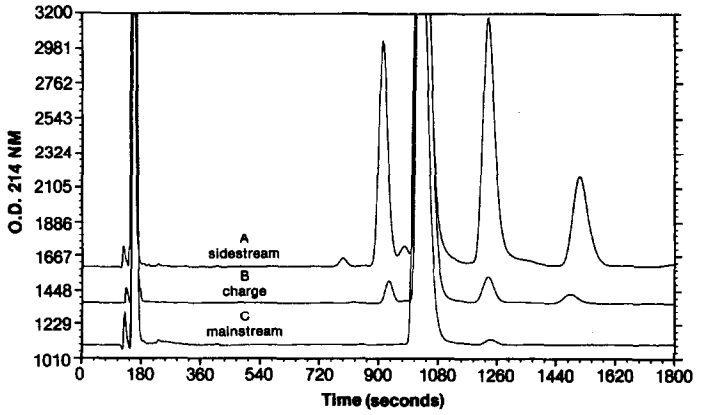

Fig.4. Analytical characterization of BHI purified by RP-HPLC (Fig. 3). Analysis conducted on a Zorbax C_8 column (30 × 0.45 cm I.D.) with pentanesulfonic acid as the mobile phase. Chromatograms: (A) BHI sidestream (purity = 50%), (B) BHI charge (purity = 91.0%), (C) BHI mainstream (purity = 98.7%).

TABLE I

COLUMN SIZES USED FOR SCALE-UP STUDIES

Column size (cm)	*Internal volume (l)*	*Type*	*Insulin load (g)**
15 × 0.94	0.010	Fixed	0.15
30 × 2.2	0.114	Fixed	1.7
50 × 5.0	0.59	Fixed	8.9
45 × 15	8.00	Axial	120
50 × 30	35.0	Axial	525
50 × 45	80.0	Axial	1200

* Assuming 15 g/l.

this series of columns, we could systematically test and modify the column packing procedures as well as the ancillary equipment required for the chromatographic operation. As the column size was increased, the quantity of insulin loaded on column was maintained at approximately 14–15 g/CV. When changing column sizes, the flow-rate and gradient volume were also changed in proportion to the volume of the column. Thus, flow-rates of 1.5 CV/h and gradient slopes of 2%/CV were used as a first approximation. Table II summarizes typical elution conditions and purification results obtained for three different column sizes using chain-derived insulin as the charge material (purity *ca.* 83%). Mainstream purities, recoveries, and volumes were consistent as the process was transferred from the lab to the pilot plant and eventually into the production facility. Minor changes in the gradient slope were necessary to obtain the desired level of purification and mainstream recovery. However, we suppose that these minor changes are due in large part to variations in the type of equipment that is being used for each scale, as well as to subtle changes in the packing, since these studies were carried out with several different lots of packing material.

Throughout the early phases of this project, we realized that if this procedure was to be implemented in a production setting, it would be necessary to purify 400–500 g of insulin in a single run. Given the loadings discussed above, a 30-l column would be required. The complexities of efficiently and reproducibly packing columns

TABLE II

SUMMARY OF SCALE-UP STUDIES

Column size (cm)	*Operation type*	*Operation conditions*			*Mainstream*		
		Flow-rate (CV/h)	*Gradient range (% acetonitrile/CV)*	*Loading*	*Purity (%)*	*Yield (%)*	*Volume (CV)*
15 × 0.94 (10 ml)*	Lab	1.6	17 to 30% (2.2%)	13 mg/ml	98.5	82	1
35 × 15 (6.2 l)	Pilot plant	1.5	13 to 30% (2%)	15 g/l	98.6	79	0.8
57 × 30 (40 l)	Production	1.4	15 to 28% (2.1%)	15 g/l	98.6	83	1.2

* Column volume

of this size with 10-μm particles are considerable. The alternative which we elected was the use of "self-packing", axial compression columns. The advantages of these systems for large-scale chromatography have been discussed previously[41–43]. Of greatest importance to us is that they provide the user with the ability to pack the columns reproducibly with chromatographic media of his/her choice. Since the units are available in a variety of sizes, initial evaluation of the process in a laboratory or pilot plant setting is possible before it is transferred to a production facility. The ease of packing and unpacking the column also makes the system attractive for pilot plant operations, where a variety of products, requiring different chromatographic media, may be processed. In both the pilot plant and production environments, we found that personnel experienced with the equipment and the packing procedure could reproducibly pack columns having over 40 000 plates/m.

Examples of production scale purification. Reversed-phase HPLC purification of BHI, derived from both the chain and proinsulin routes has been successfully conducted in a production environment. The resolving power of this procedure is perhaps best exemplified by the purification of chain-derived insulin, since it contains a number of structurally related components which can amount to 20% of the protein in the preparation. Fig. 5 shows the analytical chromatograms obtained for both the charge and the mainstream. The major insulin-like components have been identified in the charge chromatogram. The purification was accomplished on a 48 × 30 cm I.D. column, with a gradient of 17 to 30% acetonitrile over 6 CV and a flow-rate of 1.4 CV/h (0.8 l/min). The column was loaded with 500 g of BHI. The reduction in insulin-like components in this step is dramatic; the mainstream purity being 98.5% *vs.* 80% in the charge solution, with the mainstream yield being *ca.* 82%. More than 30 lots could be purified using the same packed column. Attempts to isolate insulin, at a similar level of high purity by a combination of intermediate-performance cation- and anion-exchange steps, were unsuccessful. A significant reduction in the levels of ECPs was also obtained in the RP-HPLC step. The ECP content of the charge material is typically in the range of 500–1000 ppm, while the level of ECPs in the reversed-

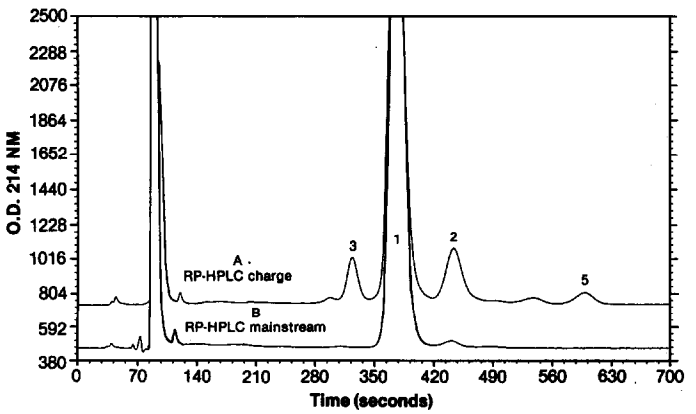

Fig. 5. Analytical characterization of chain-derived BHI, purified on a 30-cm I.D. RP-HPLC column. Analysis conducted on a Zorbax C_8 Reliance cartridge column (8 × 0.4 cm I.D.) with pentanesulfonic acid mobile phase. Chromatograms: (A) BHI charge (purity = 80.0%), (B) BHI mainstream (purity = 98.5%).

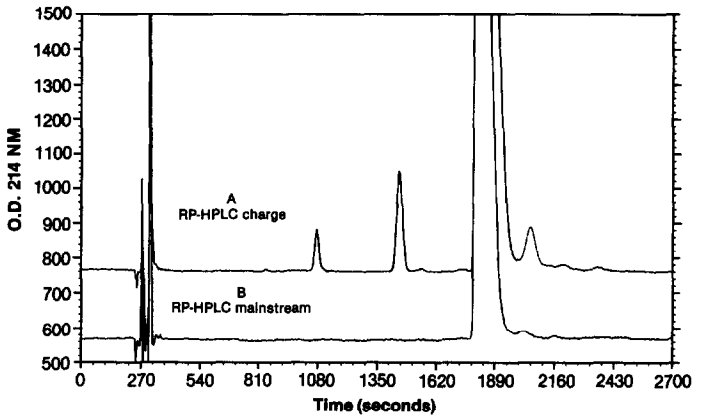

Fig. 6. Analytical characterization of proinsulin-derived BHI, purified on a 30-cm I.D. RP-HPLC column. Analysis conducted on a Zorbax C_8 Reliance cartridge column (8 × 0.4 cm I.D.) with octanesulfonic acid mobile phase. Chromatograms: (A) BHI Charge (purity = 91.5%), (B) BHI mainstream (purity = 99.1%).

phase mainstream is $\leqslant 10$ ppm. A further reduction in ECP levels is accomplished in the subsequent size-exclusion and final crystallization steps.

Proinsulin-derived BHI has also been successfully purified by RP-HPLC on a production scale. Fig. 6 shows the analytical chromatograms for a typical reversed-phase charge and mainstream. The insulin-like components present in the charge are similar to those from the chain process, but they are present at lower levels. In this example, the charge purity is *ca.* 91% and the mainstream is $\geqslant 99\%$. The purification was achieved using a 45 × 30 cm I.D. column, loaded with 500 g of BHI. The elution gradient was from 18 to 29% acetonitrile and was run over 6 CV at a flow-rate of 1.5 CV/h (0.8 l/min).

Insulin purification studies conducted with a 45-cm I.D. axial compression column have given comparable results. The use of the larger-diameter column allows over 1 kg of BHI to be processed in a single run.

Analytical characterization of final insulin crystals. Both chain- and proinsulin-derived RP-HPLC crystals were further purified by size-exclusion chromatography and zinc crystallization before being subjected to a series of assays. The key assay results for typical lots of BHI, derived from both the chain- and proinsulin-processes are summarized in Table III. Data for lots of chain-derived BHI, purified by conventional chromatographic procedures, are also included. The tests indicate a high degree of chemical purity, as evidenced by the excellent HPLC purity, and the low level of potential impurities such as ECPs and endotoxins. Note the improvement in the HPLC purity for the RP-HPLC-purified, chain-derived BHI compared to that from the conventional process. This is especially dramatic in light of the fact that this material was derived from sidestream fractions which were more highly enriched in insulin-like components prior to the reversed-phase purification step.

The final crystals were also found to be free of any siloxane products, which can result from the breakdown of the stationary phase. The presence of siloxanes in preparations of iodinated insulins, purified by RP-HPLC, has been implicated in the

TABLE III

ANALYTICAL CHARACTERIZATION OF BHI

Process	*Purification method*	*Lot*	*Biopotency (units/mg, anhydrous basis)*	*HPLC purity** *(%)*	*ECP*	*Endotoxins (E.U./mg)*	*Siloxanes (ppm)*
Chain	Conventional chromatography	A	28.4	96.1	< 4	< 0.13	–
		B	29.5	95	< 4	0.13	–
	RP-HPLC	C	30.0	98.8	< 4	< 0.18	< 10
		D	27.4	98.7	< 4	< 0.36	< 10
Proinsulin	RP-HPLC	E	28.6	99.0	< 4	< 0.13	< 10
		F	29.0	99.0	< 4	0.13	< 10

* Gradient RP-HPLC[12].

reduced biological activity observed for these preparations[25]. In the present study, the size-exclusion and final crystallization steps could remove these products from the RP-HPLC purified insulin. With this in mind, crystals obtained immediately after RP-HPLC were also analyzed for siloxane products. No siloxanes were detected in these lots during the lifetime of the packed column.

The biological potency shows that there is no difference between the activity of insulin subjected to reversed-phase purification and that obtained from the more conventional procedure. This demonstrates that with careful selection of operational conditions, insulin can be subjected to RP-HPLC purification without adversely effecting its biological potency. Successful clinical trials with insulin derived from the proinsulin process, also indicate its high degree of purity and efficacy[44].

CONCLUSIONS

The studies discussed here indicate that RP-HPLC can be an effective method for the purification of insulin. A high purity, fully active product is obtained in good yields. RP-HPLC serves as a complement to the ion-exchange and size-exclusion steps, thereby lending itself to the multimodal approach to chromatographic purification. By careful integration into the overall purification process, RP-HPLC yields a product which can be readily processed through the remaining purification steps. The process can be effectively scaled up from the laboratory, to the pilot plant, and eventually to a production environment, where multikilogram-quantities of material are produced.

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