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SEMI-PREPARATIVE HIGH-PERFORMANCE REVERSED-PHASE DISPLACEMENT CHROMATOGRAPHY OF INSULINS

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SUMMARY

A reversed-phase displacement chromatographic method has been developed for the purification of bovine and porcine insulin samples. Up to 500 mg of raw insulin could be purified on a Nucleosil C_8 analytical column using a methanol-containing phosphate buffer carrier and a cetrimide-containing displacer. A proinsulin contamination level as low as 100 ppm in the collected fractions could be achieved.

INTRODUCTION

Semi-preparative- and preparative-scale separations of both natural and synthetic oligopeptides and proteins are increasingly important for the progress of the life sciences. Efficient protein separations are generally performed by reversed-phase liquid chromatography, with either isocratic or gradient elution. Analytical-scale protein separations have been successfully scaled up to produce routinly several hundred milligram amounts of highly purified proteins. For example Rivier *et al.*[1] reported the use of 30 × 5 cm I.D. cartidge columns, packed with 460 g of 15–20 μm reversed-phase materials. These columns were eluted with binary solvent gradients to produce 99+% pure proteins of up to 44 residues. As these separations were effected in the elution mode, the solvent consumption varied in the range 4–8 l per separation.

Chromatographic separation has also been achieved by operating the column in the displacement mode[2–6]. In displacement chromatography the sample components are separated into successive bands as they are pushed forward by the continously fed displacer, which is more strongly retained than any of the sample components. Eventually, each component is compressed into a zone, the equilibrium concentration of which is determined by the adsorption strength of both the component and the displacer and the concentration of the latter. This series of zones is known as the

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displacement train. The chromatogram takes the shape of steps, the height of which is characteristic of the component and the length of which is proportional to the amount of the component present. Horvath and co-workers[7–10] demonstrated that high-performance liquid chromatographic (HPLC) hardware could be used successfully in the displacement mode to separate several hundred milligram amounts of low- and medium-molecular-weight compounds on 4 mm I.D. columns. The high throughput of columns operated in the displacement mode is due to the fact that, once the equilibrium displacement train has been formed, the remainder of the column does not improve the separation; rather, it can accommodate more sample.

Previously, we found that the retention of insulins can be easily controlled in reversed-phase systems by the addition of ion-pairing reagents[11–13]. These observations prompted us to try to combine the use of long-chain tetraalkylammonium ion-pairing reagents and the principles of displacement chromatography for the preparative purification of raw insulin. Long-chain tetraalkyl ammonium cations were expected to compete effectively with insulins and other proteins for the hydrophobic, hydrophilic and negatively charged ionic adsorption sites of silica-based reversed-phase packings. As both the ionic character (permanent ions) and molecular weight of the tetraalkylammonium cations differ significantly from those of the proteins, they can be removed from the pure protein fractions by either simple ion-exchange or size-exclusion methods. The results of the first phase of this project are reported in this paper.

EXPERIMENTAL

Equipment

Displacement separations were carried out with a Varian LC 5020 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.), equipped with a Model 7010 manual 5.4-ml loop sampling valve (Rheodyne, Cotati, CA, U.S.A.) and a 250 × 4.6 mm I.D. stainless-steel column (Chrompack, Middelburg, The Netherlands). The column was thermostated at 30°C with a water-jacket. An LC55 variable-wavelength UV detector (Perkin-Elmer, Norwalk, CT, U.S.A.), operated at 295 nm and an A25 dual-channel recorder (Varian) were used to record the displacement chromatograms. The columns were packed with spherical Nucleosil C8 octyl silica (Macherey, Nagel & Co., Bad Durkheim, F.R.G.), particle size 5 μm, by Bioszeparacios Technika (Budapest, Hungary).

Analytical HPLC separations were effected by an M6000 pump, U6K injector (Waters Assoc., Milford, M.A., U.S.A.) and a 250 × 4.6 mm I.D. LiChrosorb RP-18 (10 μm) column (Merck), thermostatted at 30°C with a water-jacket. An LC55 variable-wavelength UV detector (Perkin-Elmer), operated at 225 nm, and a A25 dual-channel recorder (Varian) were used to record the chromatograms. The conditions used for the analytical insulin separations were described in detail in refs. 11 and 13.

Materials

Bovine and porcine insulin samples were obtained from Gedeon Richter Works (Budapest, Hungary). NaH_2PO_4, H_3PO_4, sodium bromide, tetrabutylammonium bromide, cetyltrimethylammonium bromide (cetrimide), 4-(2-pyridylazo)resorcinol

(PAR), methanol and acetonitrile were analytical-grade reagents from Reanal (Budapest, Hungary).

The carrier was methanol–50 m*M* phosphate buffer (pH 2.2) (5:95, v/v). Displacer solutions were prepared by dissolving the respective displacer in the carrier. The regenerant was methanol–50 m*M* phosphate buffer (pH 2.2) (94:6, v/v).

The eluent used for the HPLC analysis of the collected displacement fractions was prepared by adding 450 g of methanol, 25 ml of 1 *M* H_3PO_4, 25 ml of 1 *M* NaH_2PO_4 and 6.64 g of tetrabutylammonium bromide to a 1000-ml volumetric flask and diluting to volume with distilled water.

The insulin samples (50–500 mg) were weighed into 5-ml screw-capped vials and were dissolved immediately before displacement chromatography in a mixture of 1:4 ml of carrier, 0.2 ml of 1 *M* H_3PO_4, and 0.2 ml of 1 *M* NaH_2PO_4 solution, added in this order.

Methods

The three solvent reservoirs of the LC 5020 chromatograph were filled with the carrier, displacer and regenerant solutions. First, the system was flushed with the carrier, then the insulin sample was injected by pumping the carrier at 0.1 ml/min. The pump was stopped, the bypass valve just upstream of the injector was opened and the system was flushed with the displacer at a high flow-rate. The pump was stopped again, the bypass valve was closed and the injection valve was switched back to the load position. Then, the LC 5020 pump was restarted to deliver the displacer at the required low flow-rate (0.1 or 0.2 ml/min). The displacement chromatogram was recorded at 295 nm. As insulin emerged from the column, fractions of 0.2–1.5 ml were collected manually for subsequent HPLC analysis.

Once all insulin had left the column, the pump was stopped, the bypass valve was opened and the system was flushed with the carrier at a high flow-rate. Then the bypass valve was closed and the carrier was pumped through the column at a flow-rate of 1.5 ml/min. A 30-min linear gradient was applied between the carrier and the regenerant (0–100%). A 100-ml portion of the regenerant was pumped through the column, followed by a linear reverse gradient in 15 min back to the carrier, and then a further 50-ml portion of the carrier to re-equilibrate the column. This procedure led to very reproducible displacement chromatograms.

Following displacement chromatography, each collected fraction was analysed by HPLC for its insulin content and its contaminants[11,13]. Except for a very small peak close to the dead volume, all contaminants were eluted after the peak of bovine and porcine insulin. All contaminants showed biological activity similar to that of insulin, although the response was slightly weaker. The contaminants are assumed to be desamido-insulins and proinsulins. No special effort was made to determine their identity positively. An empirical purity factor characterizing the efficacy of the displacement separation was defined as the ratio of the area of the insulin peak to the sum of the areas of the contaminant peaks.

A spectrophotometric method, based on the formation of coloured mixed complexes of long-chain tetraalkylammonium cations, PAR and vanadium(V)[14,15], was used to monitor the breakthrough of the tetraalkylammonium displacer agent.

RESULTS AND DISCUSSION

Selection of the displacement system

As in displacement chromatography the concentration of the sample in the mobile phase is characteristically (and advantageously) high, the carrier must be a good solvent for the sample. On the other hand, it has to ensure that on injection the sample is adequately retained at the top of the column. It was known from our previous studies[13] that insulin is retained excessively on reversed-phase columns when the methanol concentration of the eluent is below 35%(w/v). On the other hand, high cetrimide concentrations require the use of sufficiently high methanol concentrations. Also, a certain minimum methanol concentration is necessary in order to maintain the solvated state of the alkyl chains of the packing. Therefore, as a compromise, a 5%(v/v) methanol concentration was selected for the carrier.

The displacement chromatogram of 150 mg of bovine insulin is shown in Fig. 1 (run 1). The cetrimide concentration in the displacer was 25 m*M*; the flow-rate was 0.2 ml/min. A regular displacement chromatogram with sharp front and tail ends was obtained. There was a small elution peak at the leading edge.

This chromatogram indicates that the hardware, the phase system and the general operating conditions (concentrations, flow-rate and temperature) were suitable for the reversed-phase displacement chromatography of insulin.

In order to establish whether an actual separation of bovin insulin and its contaminants could be achieved, 0.5-ml fractions were collected between 35 and 55 ml. These fractions were analysed by HPLC and the displacement chromatogram was reconstructed from the results of the HPLC analysis, as shown in Fig. 2.

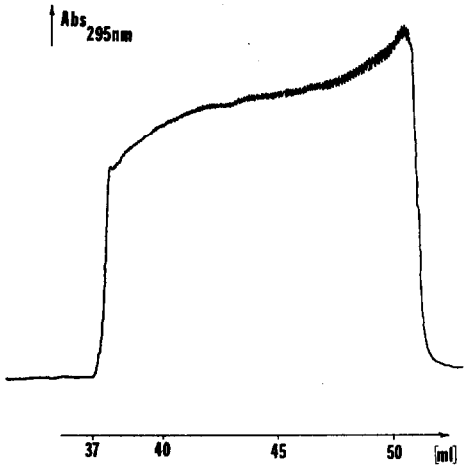


Fig. 1. Displacement chromatogram of 150 mg of bovine insulin (run 1). Displacer: 25 m*M* cetrimide in carrier.

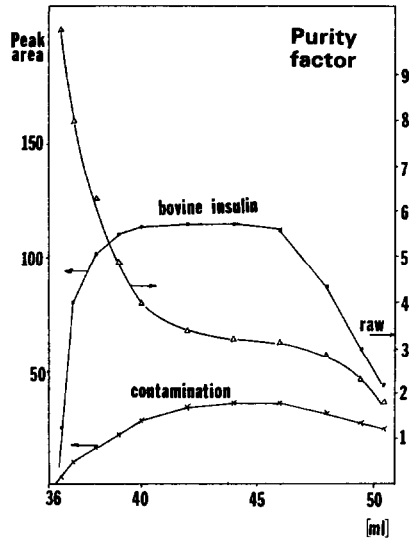


Fig. 2. Displacement chromatogram reconstructed from HPLC analysis of run 1 fractions. Left ordinate, peak area (at 2.25 nm) of insulin and contaminants (arbitrary units); right ordinate, purity factor (△), defined under *Methods*. Purity factor of the raw insulin sample, 3.18. Note the lowered impurity level in the 36-ml to 43-ml fractions and the accumulation of contaminants in the 46-ml to 51-ml fractions.

Insulin was recovered in a total volume of 15 ml, starting at 37 ml. Tetraalkylammonium cations could be detected only in the fractions collected after 51 ml. This means that cetrimide is a suitable displacer; it does not contain low-molecular-weight ionic contaminants that may interfere with the separation, and also it provides a sharp front, leading to good product recovery.

From the displacement chromatogram we can calculate the characteristic adsorption data of the system. The weight of the packing in the column (on a dry basis) is 2.2 g. The breakthrough volume of cetrimide is 51 ml and the mobile phase cetrimide concentration is 25 mM; consequently the amount of cetrimide adsorbed on the column is 1.28 mmol, and its equilibrium surface concentration is *ca.* 0.57 mmol/g. This results in a cetrimide distribution coefficient of $K = 2.28 \cdot 10^{-2}$ (mol/g)/(mol/l), *i.e.* l/g.

As in a well developed displacement train the distribution coefficients of all components are equal, the distribution coefficient of insulin is also $2.28 \cdot 10^{-2}$ l/g. As a first-order approximation, we can assume that all the sample loaded on to the column is insulin. As this material was recovered in a total volume of approximately 14 ml, the average equilibrium mobile phase insulin concentration is 10 mg/ml. Hence the stationary phase concentration of insulin in the developed train is 0.23 g of insulin per gram of packing. This means that only 0.66 g of the packing is covered with insulin as the leading edge of the insulin zone reaches the end of the column. This corresponds to a column utilization of 29%; consequently, the maximum amount of insulin that can be loaded on to the column is approximately three times the present amount, or 510 mg.

The concentration profiles of insulin and its contaminants are shown in Fig. 2. It can be seen that the purity factor exceeds the original value (3.18) in the first 7 ml, and that the contaminants are more concentrated in the last 5 ml. This means that the separation selectivity of our displacement system is sufficient to effect at least a partial separation of insulin and its contaminants (*i.e.*, the adsorption isotherms of insulin and its contaminants are different from each other). The separation cannot be improved by selecting another displacer, because it would have no effect on the shape and location of the adsorption isotherms of insulin and its contaminants.

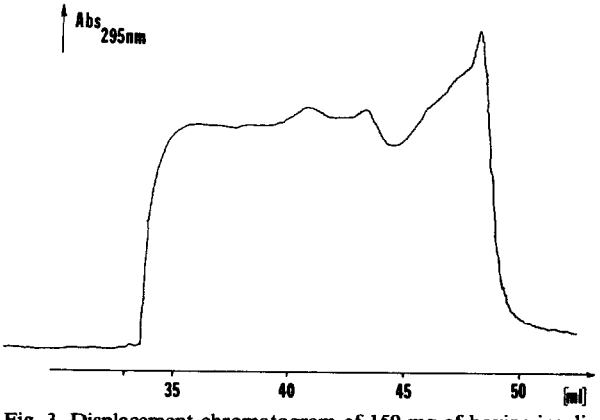
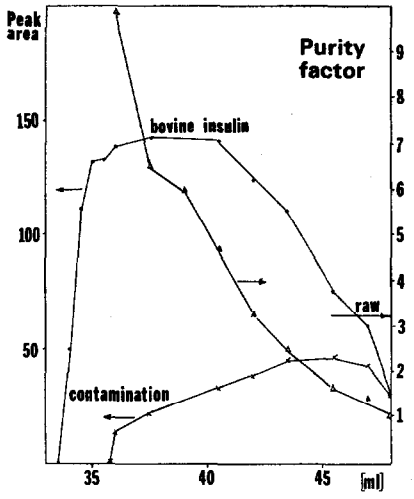


Fig. 3. Displacement chromatogram of 150 mg of bovine insulin (run 2). Conditions as in run 1, except flow-rate, 0.1 ml/min.



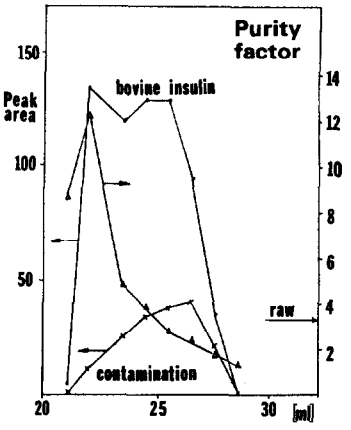


Fig. 4. Displacement chromatogram reconstructed from HPLC analysis of run 2 fractions. Note the improved purity in the first half of the chromatogram and the accumulation of contaminants in the last fractions.

Fig. 5. Reconstructed displacement chromatogram of 130 mg of bovine insulin (run 3). Conditions as in run 2, except displacer concentration, 50 m*M* cetrimide. Note that no pure insulin is obtained in this instance.

Therefore, the effects of the other separation variables, flow-rate, displacer concentration and sample load were surveyed.

Effects of flow-rate

The displacement chromatogram of 150 mg of bovine insulin (run 2) is shown in Fig. 3. The analytical conditions were as in run 1, except that the flow-rate was 0.1 ml/min. The injected material was again recovered in 15 ml and the shape of the displacement chromatogram is similar to that of run 1, except that more structure can be seen in the last part of the chromatogram.

The reconstructed displacement chromatogram is shown in Fig. 4. The extent of purification is much improved over run 1. No contaminant can be detected in the first 3 ml (20% of the fractions) and the purity factor in 57% of the fractions is larger than in the raw insulin sample. There is a large accumulation of contaminants in the last 2 ml: the areas of the insulin peak and the contaminant peaks are equal. The improvement in the separation is due to reduced band broadening and mixing at the zone boundaries.

Effects of displacer concentration

The effects of displacer concentration were tested at a higher and a lower level than in run 2. The reconstructed displacement chromatogram of 130 mg of bovine insulin with 50 m*M* cetrimide (run 3) is shown in Fig. 5 and, that of 140 mg of bovine insulin with 10 m*M* cetrimide (run 4) is shown in Fig. 6. The other experimental conditions were the same as in run 2. Calculated cetrimide and insulin concentrations and partition coefficients are given in Table I.

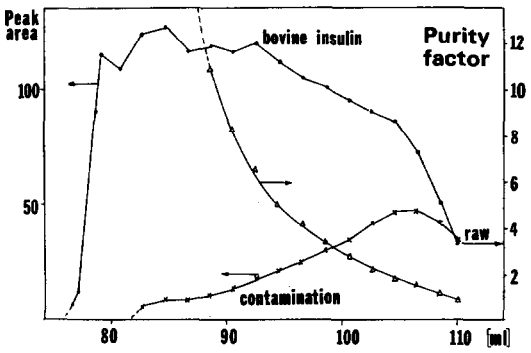


Fig. 6. Reconstructed displacement chromatogram of 140 mg of bovine insulin (run 4). Conditions as in run 2, except displacer concentration, 10 m*M*. Note the presence of pure insulin fractions and the improved purification in the first half of the chromatogram.

Fig. 5 shows that, although the column can be loaded with more insulin than in run 2, the extent of purification is not as good as in run 2. There are no entirely contaminant-free insulin fractions, and the contaminants are less concentrated in the final fractions.

When the displacer concentration was decreased to 10 m*M* (run 4, Fig. 6) the permissible maximum load was lower but the extent of purification that could be achieved was much higher. Not only was there a substantial contaminant-free insulin fraction, but also the middle fractions were purer and the contaminants were concentrated in the last fractions.

Effects of increased sample load

The reconstructed displacement chromatogram of 260 mg of bovine insulin (run 5) is shown in Fig. 7. The experimental conditions were the same as in run 4, except that the sample load was almost doubled. Insulin was recovered in 66 ml, with

TABLE I

CETRIMIDE AND INSULIN CONCENTRATIONS AND PARTITION COEFFICIENTS CALCULATED FROM DISPLACEMENT RUNS 2–4

C_M = concentration of cetrimide in displacer (m*M*); V_c = breakthrough volume of cetrimide (ml); C_S = stationary phase concentration of cetrimide (mmol/g); K_c = distribution coefficient of cetrimide (l/g); K_i = distribution coefficient of insulin (l/g); m_i = amount of insulin injected (mg); V_i = volume of carrier containing all recovered insulin (ml); C_M^i = equilibrium mobile phase concentration of insulin (g/l); C_S^i = equilibrium stationary phase concentration of insulin (g/g); coverage = amount of packing covered by insulin (g and %); load = maximum permissible sample load on column (mg of insulin).

Run No.	*Cetrimide*				*Insulin*							
	C_M *(mM)*	V_c *(ml)*	C_S *(mmol/g)*	K_c *(l/g)*	K_i *(l/g)*	m_i *(mg)*	V_i *(ml)*	C_M^i *(g/l)*	C_S^i *(g/g)*	*Coverage*		*Load (mg)*
										g	*%*	
4	10	114	0.51	5.09	−2	140	38	3.7	0.19	0.75	33	420
2	25	51	0.57	2.28	−2	150	15	10.0	0.23	0.66	29	510
3	50	27	0.60	1.20	−2	130	6.3	20.6	0.25	0.52	23	557

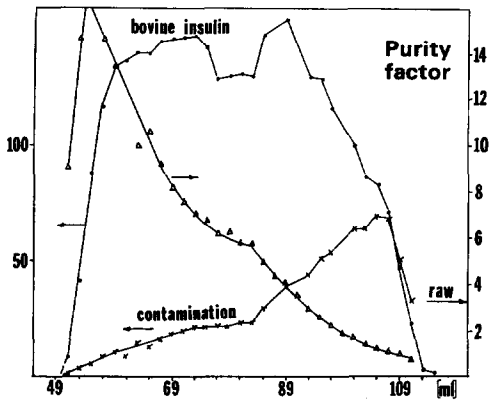


Fig. 7. Reconstructed displacement chromatogram of 260 mg of bovine insulin (run 5). Conditions as in run 4. The extent of purification is the same as in run 4. (△) Purity factor.

an average concentration of 3.9 mg/ml, a value very close to that in run 4. Cetrimide breakthrough was at 114 ml, the same as in run 4. It can be seen that increased sample loading did not diminish the quality of the separation.

Purification of mixed bovine and porcine insulin

The reconstructed displacement chromatogram of a mixture of 85 mg of porcine insulin and 90 of bovine insulin is shown in Fig. 8 (run 6). The operating conditions were the same as in run 5. The peak-area values of porcine and bovine insulin and their contaminants were plotted separately.

It is striking that procine insulin leaves the column first and bovine insulin second. This order is the opposite of what is usually observed in reversed-phase elution chromatography, *i.e.*, bovine insulin followed by human and porcine insulin[11–13,16–19].

It is known that in reversed-phase liquid chromatography both small polar amino compounds[20–22] and large oligopeptides[23] are retained by a sensitive balance

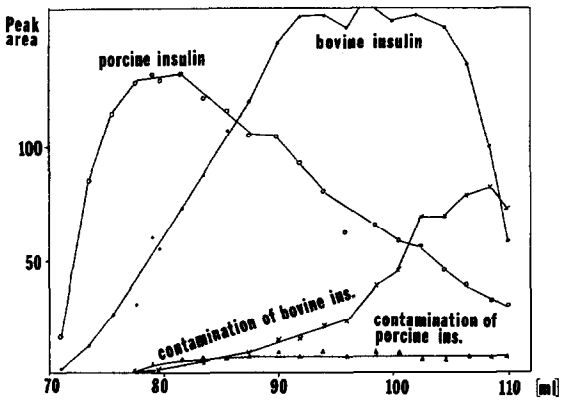


Fig. 8. Reconstructed displacement chromatogram of a mixture of 85 mg of porcine and 90 mg of bovine insulin (run 6). Conditions as in run 4.

of hydrophobic and hydrophilic forces and that their retention varies with the organic modifier content according to a minimum curve. This behaviour has been termed the "dual-retention mechanism"[20].

McLeod and Wood[18] stated that porcine insulin is more hydrophilic than bovine insulin, because there is a threonine unit in position 8 of chain A. In eluents with intermediate organic solvent concentrations (the general condition for the elution of insulins) the retention of threonine-substituted insulins can be either larger (as in the case of human and porcine insulins[18]) or smaller (as Terabe *et al.*[24] found for the B30 substituted threonine analogue of bovine insulin and bovine insulin itself), depending on the hydrophilic/hydrophobic balance of the packing and the mobile phase.

As the carrier used in our displacement system was lean in methanol, and as the partition coefficients of both cetrimide and insulin seem to be the same on both the C_8 and C_{18} packings[25], *i.e.*, their value is controlled more by the hydrophobicity of the stationary phase, it is not unexpected that the more hydrophilic porcine insulin leaves the column first.

It can also be seen in Fig. 8 that the contaminant of porcine insulin (desamido porcine insulin) leaves the column at the same time as pure bovine insulin. Hence the proposed displacement scheme is unsuitable for the single-run purification of mixed bovine and porcine insulin batches. Fortunately, both porcine and bovine proinsulins leave with the cetrimide front and are well separated from the insulins.

CONCLUSIONS

A displacement chromatographic method has been developed for the purification of raw bovine and porcine insulin samples. Using a 5-μm Nucleosil C_8 packing, a 5% methanol in phosphate buffer carrier and a 10–25 m*M* cetrimide-containing 5% methanol–phosphate buffer as displacing agent, insulin samples as large as 260 mg could be succesfully handled in a single run on 4.6 mm I.D. columns. The fractions collected during the first quarter of the run contained monocomponent insulin, those collected in the second quarter were considerably purer than the raw material, whereas samples collected in the second half of the run could be pooled and reprocessed. The concentration of proinsulin in the purified fractions was below the 100 ppm target level. We are currently working on the scale-up of this displacement chromatographic method.

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