Influence of operating parameters on the preparative gradient elution chromatography of insulins

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ABSTRACT

The mass-overloaded separation of bovine and porcine insulins has been studied in the reversed-phase gradient elution mode. Strong solute–solute displacement effects have been found, which are related to the efficiency of the column used. Low flow-rates and small particle diameters maximise the displacements, as well as improve the resolution between the parent insulin and its desamido contaminant. The gradient slope did not substantially affect the separation between the parent insulins, but, due to the relative "S" values of the solutes, an increase in gradient slope improved the separation of the parent insulin from its desamido compound. An optimum pore size of 150 Å was found for the insulins. Experiments to optimise the loadability were not carried out, but a recovery of 90% at a purity of 99.5% was obtained with a loading of 12 mg/g of porcine insulin.

INTRODUCTION

The isolation of peptides and proteins by preparative reversed-phase chromatography is becoming a preferred route toward their purification. During the past two years, studies of solute–solute interactions in the mass-overloaded separations of proteins have demonstrated that strong displacements can occur between the components of the samples. The separation of cytochrome c from lysozyme or RNAse [1] by preparative gradient elution chromatography has been shown to be characterised by strong solute–solute displacements. The question of the occurrence of such strong displacements between more closely related proteins was not addressed. Although displacements during the elution chromatography of proteins are not well known, several separations of proteins by displacement chromatography have been reported. Papers by Huang and Horváth [2] and by Subramanian *et al.* [3] have demonstrated that very strong displacements can be seen under the right conditions. In many of these cases, the structural difference between the proteins is marked, and it is not clear if very closely related proteins would demonstrate the same effects.

Vigh *et al.* [4] have described the displacement chromatography of bovine and porcine insulins. These have very similar structures and appear to be more difficult to separate, at least under the reversed-phase conditions reported. The yields and purity of materials isolated from these experiments were rather poor, and displacement trains were not apparently well established. It is not clear if the problem lay in the choice of cetrimide as a displacer, poor isotherms or too high a load and insuffficient column length.

The elution chromatography of insulins is well documented, and reversed-phase preparative liquid chromatography has been carried out on a number of insulin variants [5,6]. Probably the most important of these is the production-scale purification of biosynthetic human insulin for use in the treatment of diabetes mellitus [7]. This reversed-phase purification was carried out on a large scale using high-efficiency columns, showing approximately 40 000 plates/m. The purity of the insulin was raised from 83 to 98.6% with a recovery of 82% at a sample loading of 21 mg insulin/g of packing material. It was not explicitly shown that displacement effects occurred. From inspection of the analytical chromatograms, however, one can deduce that they

were probably important since the impurities eluted very closely to the major peak and would otherwise have been engulfed by it at the loads described.

The work reported in this paper was carried out to study the effects of operating conditions — gradient slope and initial composition, flow-rate, particle size, pore diameter, etc. — on the interactions between solutes which are structurally similar. The same insulin variants, bovine and porcine insulins, were chosen for this work as had been used in the earlier displacement work. This choice reflected the availability of standard materials rather than a conscious attempt to compare the techniques of gradient elution and displacement chromatography. Nevertheless, it was felt that the results may be relevant to the continuing discussions upon the relative merits of the techniques.

EXPERIMENTAL

Equipment

A HP1090 L liquid chromatograph (Hewlett-Packard, Avondale, PA, USA), fitted with a preparative autoinjector and a diode array detector was used for this work. Data storage and processing were performed using a HP 3365 Chem Station data system.

Chromatography

Columns. Kromasil 100 10 C_8 and 150 10 C_8 columns (Eka Nobel, Surte, Sweden, 10 μm, 100 and 150 Å pore size respectively), 25 cm × 4.6 mm I.D. and a range of Matrex 250 Å C_8 columns (Amicon, Danvers, MA, USA), of 6.5, 20 and 50 μm particle size, 10 cm × 4.6 mm I.D. were obtained from the manufacturer. Zorbax® PRO-10 Protein Plus (DuPont, Wilmington, DE, USA; 10 μm, 300 Å pore size) and Poroquartz 200 C_{18} (10 μm, 200 Å pore diameter) (Moscow Institute of National Economy, Centre of Applied Liquid Chromatography, Moscow, USSR) were packed into columns (15 cm and 25 cm × 4.6 mm I.D., respectively) by a downward slurry technique.

Chemicals and mobile phases. Acetonitrile was obtained in HPLC grade from EM Science (Gibbstown, NJ, USA). Trifluoroacetic acid (TFA) was obtained from J. T. Baker (Phillipsburg, NJ, USA). Protein standards were from Sigma (St. Louis, MO, USA). HPLC-grade water was prepared with a Na-

nopure unit (Barnstead/Thermolyne, Dubuque, IA, USA).

Analysis of fractions. Because the deamidation of insulins is promoted by low pH, the fractions from the preparative separations were either collected into a pH 7 buffer or (more usually) were stored at 4°C between collection and analysis. No evidence for the decomposition of insulins in the fractions was found.

RESULTS AND DISCUSSION

The structures of bovine and porcine insulins are closely similar and the chromatographic separation of these compounds is expected to be difficult. A conventional acetonitrile–0.1% trifluoroactic acid gradient was used throughout, since it was known that such a solvent system would allow the elution of the insulins and also the dissolution of the samples to the concentrations required for the study. A low-pH solvent was used, despite the possibility of deamidation of the insulins, because the value of the isoelectric point of insulin ($pI \approx 5.5$) means that operation at a significantly higher pH would limit the solubility. During this study, no attempt was made to optimise the mobile phase components. The reason for this was because a difficult separation was required for the study; too high a selectivity would negate part of the reason for carrying out the work.

Preparative gradient design

In order to design the gradient to effect the separation, it was desirable to determine the basic gradient parameters. These are the slope of a plot of log capacity factor *vs.* solvent composition (S) and the capacity factor of the solutes in water (k'_w) [8]. From these data, it is possible to calculate the retention time of the solutes using any other gradient profile. A commercial software package (DryLab G; LC Resources, Lafayette, CA, USA) was used for the calculations.

Measurements of retention times of the solutes were made on Zorbax Pro-10 Protein Plus under the conditions described in Table I. The S and log k'_w values determined are also shown in that Table. The predictions from the software suggested that an isocratic composition of 30% would give a good resolution of the components. This was refined ex-

TABLE I

GRADIENT PARAMETERS FOR BOVINE AND POR-CINE INSULINS

Experimental conditions: column, Zorbax Pro-10 Protein Plus, 25 cm × 4.6 mm I.D.; gradient, 10 to 60% acetonitrile in 0.1% aqueous TFA; gradient duration: (a) 30 min, (b) 90 min; flow-rate, 1.0 ml/min; detection wavelength, 230 nm; sample, 10 μl of 1 mg/ml solution of bovine and porcine insulins in 10% aqueous acetonitrile containing 0.1% TFA.

Solute	S	Log k'_w
Bovine insulin	14.9	5.31
Porcine insulin	15.23	5.48
Desamidoporcine	14.92	5.45

TABLE II

RECOVERIES OF BOVINE AND PORCINE INSULINS

(a) Isocratic conditions: loading, 2 min at 10% acetonitrile in 0.1% aqueous TFA; gradient to starting conditions; 10 to 90% acetonitrile in 0.1% aqueous TFA over 10 min; separation conditions, isocratic, 29% acetonitrile in 0.1% aqueous TFA; flow-rate, 0.5 ml/min; detection wavelengths, 230 and 290 nm; sample, 250 μl of a solution 5 mg/ml of each of bovine and porcine insulin. (b) Gradient conditions: gradient to starting conditions, 10 to 27% acetonitrile in 0.1% aqueous TFA over 10 min; separation conditions; 27 to 31% acetonitrile in 0.1% aqueous TFA over 28 min; other conditions as (a).

Conditions	Ratio	Bovine		Porcine	
		Purity (%)	Recovery (%)	Purity (%)	Recovery (%)
b	1:1	100	91	99.5	83
a	1:1	100	95	99.5	82
b	1:9	100	61	99.5	90
b	9:1	100	98	100	96

perimentally to 29%, and a gradient from 27 to 31% acetonitrile was selected. Following the recommendations arising from an earlier study of the separation of proteins by preparative reversed-phase chromatography [9], the sample was loaded at a low solvent strength to avoid the problem of displacement of acetonitrile by the proteins during their adsorption and the consequent elution of part of the sample. A steep gradient from the 10% loading composition to the initial composition of the elution gradient was used. Because the resolution at 1 ml/min was small, the chromatography was performed at 0.5 ml/min, which was seen to improve the separation by reduction of the band width of the insulins by increasing the column efficiency.

Effect of gradient slope

Preparative separations of the insulins were performed both isocratically at 29% acetonitrile and by gradient, from 27 to 31% acetonitrile in 28 min, using the Pro-10 Protein Plus column under conditions shown in Table II. A sample load of 2.5 mg of a 1:1 mixture of the insulins was introduced at 10% acetonitrile in the mobile phase. This was increased over 10 min (later work showed that a step gradient was just as effective) to the desired elution composition. Fractions were collected through the peak envelopes and these were subsequently analysed for content of the components of the mixture. Fig. 1 shows the gradient elution chromatogram together with the distribution through the peak of the individual components, reconstructed from the results

of analysis of the fractions. Fig. 2 shows similar data from the isocratic experiment. In both chromatograms, the trailing edge of the bovine insulin peak does not follow the same profile as that of the porcine insulin. The tail shows a distinct break at the point at which the porcine insulin starts to elute, and rapidly falls to the baseline. If this displacement were not observed, the bovine insulin would tail to
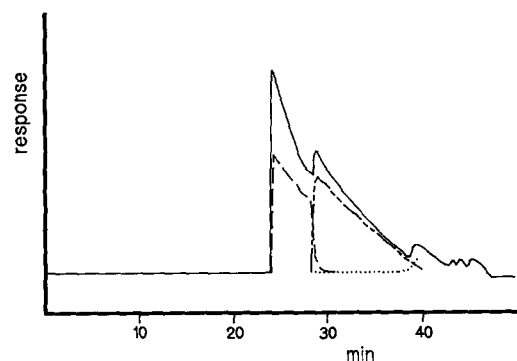

Fig. 1. Gradient chromatogram of a 1:1 mixture of bovine and porcine insulins, 2.5 mg total load. Solid line: chromatogram. Dashed line: bovine insulin; dashed/dotted line: porcine insulin; dotted line: desamidoporcine insulin. Conditions as Table II, conditions b.
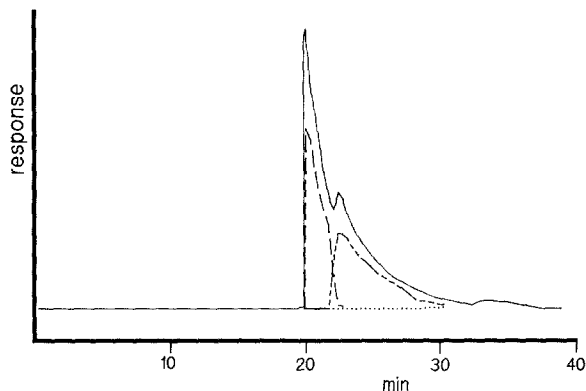
Fig. 2. Isocratic chromatogram of a 1:1 mixture of bovine and porcine insulins, 2.5 mg total load. Key as Fig. 1. Conditions as Table II, conditions a.

Fig. 3. Gradient chromatograms of 9:1 and 1:9 mixtures of bovine and porcine insulins, 2.5 mg total load. Gradient conditions as Fig. 1. (a) Bovine–porcine insulins (9:1) gradient chromatogram; (b) bovine–porcine insulins (1:9) gradient chromatogram; key as Fig. 1.

the retention time of an analytical scale load and would substantially contaminate the porcine insulin. At the same time, of course, it would itself be substantially contaminated by the porcine insulin with which it was co-eluting. In the isocratic separation, where the tail of the porcine insulin peak is extremely long, the extent of peak overlap would be very great. The displacements are similar to those seen in the preparative separations of other proteins [1,9] and mean that the purity and recovery of the components are higher than would at first be expected.

Combinations of the collected fractions were calculated which gave a product with a purity of at least 99.5%. The recoveries of the insulins were determined for these combinations. These are shown in Table II, along with data calculated from similar gradient runs using the same total load but 1:9 and 9:1 mixtures of the two insulins. Chromatograms arising from these last two experiments are shown in Fig. 3, along with the distribution of each component through the main peak envelope, derived again from the results of the analysis of the fractions collected during the experiment. These show the same strong displacements as seen in the 1:1 mixtures, and also demonstrate the absence of any "tag-along" effect between the insulins.

There was little difference between the recoveries of the isocratic and gradient experiments. This is consistent with both theory [10] and results reported earlier [9]. The results for the experiments using 1:9 and 9:1 mixtures were at variance with results of
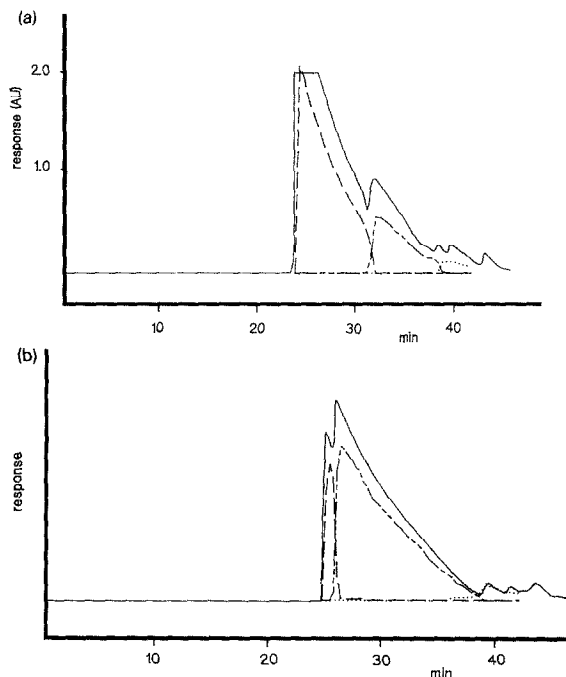
computer simulations in isocratic chromatography [11], where the displacement effects gave a better recovery of the first eluted component when it was present at lower concentration. This is because there is no tag-along effect between the bovine and porcine insulins; the displacements maintain the separation between them. Inspection of the two chromatograms reveals that the zones of overlap between the peaks are similar for the 1:9 and 9:1 mixtures. When the bovine insulin is the minor component, the overlap with the high concentration front of the porcine insulin peak means that it is substantally contaminated with the later eluting component. When the porcine insulin is the minor component, it interacts only with the low-concentration tail of the bovine insulin, is thus less contaminated and the recovery is higher.

The influence of gradient slope on separations using higher sample loads was also investigated. This work was performed on a smaller column, 10 cm × 4.6 mm I.D. packed with 6.5-μm C_8 particles of

250-Å pore diameter, and with twice the load used earlier. Allowing for the higher surface area of the smaller pore, irregular packing material, this increased the effective load on the column by a factor of around 4. The increase in load was expected to have a deleterious effect upon the recovery at a given purity of insulin. In addition, this separation was performed using a mixture of less pure insulin standards which contained appreciable quantities of both of the desamido compounds.

Two gradient experiments were carried out, using 10- and 30-min gradient times respectively, as detailed in Table III. Fractions were collected through the main peak envelopes and were analysed using the same column, but under isocratic conditions (30% acetonitrile) which allowed a good resolution of all of the components. The results of this analysis are shown in Fig. 4, which shows the profiles of the individual components through the peak envelopes for the two gradients. Table III also shows the purity and recoveries achieved under these conditions. Despite the higher load, the recovery of product at high purity remained acceptable, especially for the earlier eluted bovine insulin. Interestingly, the separation with the steeper gradient gives better recoveries than the other. This can be understood by reference to the S values of the insulin versus its desamido impurity. Earlier work has suggested that the separations of proteins are dependent not only upon the gradient parameters but also upon their S values [1]. When the S values of two components are equal, the separation should be independent of the gradient slope. When they are unequal, the effects of the slope will depend upon which component has the greater value of S. If the first eluting component has the greater S value, a steeper gradient will improve the separation, since the components elute at a higher effective solvent strength. This is the case here, in that the desamido impurities have lower S values than the parent compounds (experiments not reported here indicate that the desamido bovine insulin has a lower S value than its parent). Thus, the separation of both the bovine and porcine insulins from their desamido impurities will be improved by the steeper gradient. It might be expected that the separation between the desamido bovine insulin and the porcine insulin would be degraded at the higher gradient slope; this separation is probably maintained because of the displacement effects.

TABLE III

EFFECT OF GRADIENT SLOPE ON RECOVERIES OF BOVINE AND PORCINE INSULINS

Column, Matrex 250 C$_8$ (6.5 µm), 10 cm × 4.6 mm I.D.; loading, 2 min at 10% acetonitrile in 0.1% aqueous TFA; gradient, step to 29% acetonitrile, 29 to 33 % acetonitrile in 0.1% aqueous TFA; gradient durations, 10 and 30 min; flow-rate, 1 ml/min; sample, 250 µl of 10 mg/ml of each of bovine and porcine insulins in 0.1% aqueous TFA containing 10% acetonitrile.

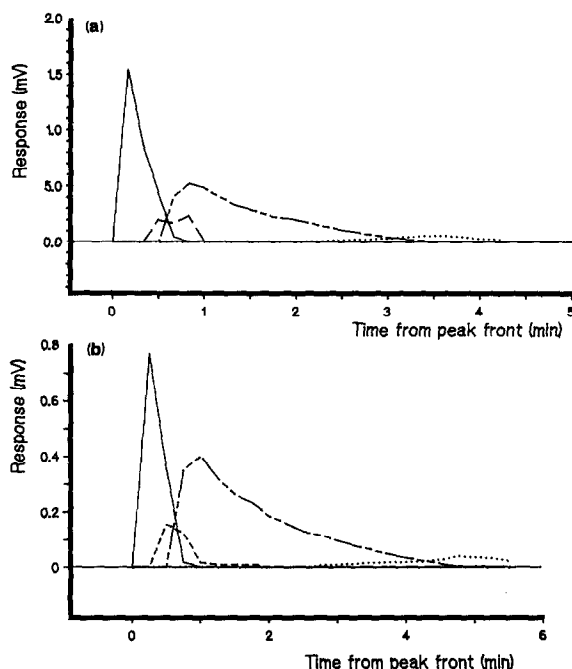| Gradient run time (min)	Bovine		Porcine	
	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)
10	100	83.5	100	58.4
	93.3	98.5		
	78.8	100		
30	100	67.4	100	18.9
	87.8	98.5	97.6	67.5
	64.4	100	97.0	76.0
			91.8	81.5

Fig. 4. Influence of gradient slope on the separation of bovine and porcine insulins, load: 2.5 mg each. Reconstructed chromatograms from fraction analysis; (a) Conditions as Table III, conditions a; (b) as (a) but final gradient duration was 30 min. Column: 10 × 0.46 cm I.D. Matrex 250 C$_8$, 6.5 µm. —— = Bovine insulin; – – = desamido bovine insulin; – – – – = porcine insulin; ···· = desamido porcine insulin.

Effect of flow-rate

The column efficiency in the chromatography of proteins is strongly influenced by the flow-rate in the system due to the very low values of the diffusion coefficients of these large molecules. The separation of porcine insulin from its desamido impurity was studied at two disparate flow-rates in order to investigate the effects of changes in efficiency. In order to maintain all other parameters constant, the gradient slopes in terms of % change per ml were maintained constant between the two runs whilst the flow-rates were changed from 1 ml/min to 0.1/ min. Other conditions are described in Table IV. Fig. 5 shows the chromatograms, reconstructed from analysis of fractions taken through the peak envelope. The improvement in the separation caused by operation at the low flow-rate is clear. The front-running impurity has a much narrower band width at the low flow-rate, reflecting the narrower displacement zone at the higher column efficiency. In addition, the separation between the main peak and that of the desamido insulin is also improved at the lower flow-rate. When the separation is performed at 1 ml/min, there appears to be a pronounced "tag-along effect" of the desamido compound. Reduction of the flow-rate (and the consequent increase in efficiency) reduces this quite
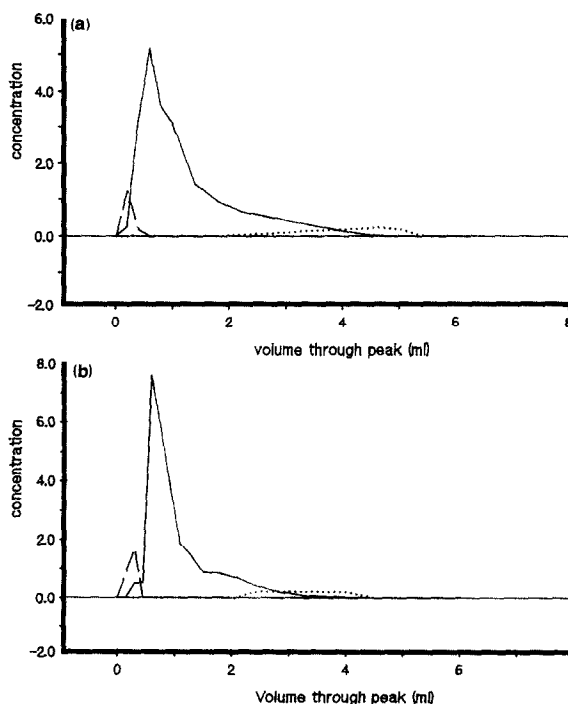

Fig. 5. Influence of flow-rate upon the purification of porcine insulin. Sample load 30 mg. Flow-rates: (a) 1 ml/min; (b) flow-rate: 0.1 ml/min. Other conditions as in Table IV. – – – = Unknown impurity; ——— = porcine insulin; ···· = desamido porcine insulin. y-Axis: arbitrary units related to peak areas.

TABLE IV

RECOVERIES OF PORCINE INSULIN AT 1 AND 0.1 ml/ min

Column, Kromasil 100 C_8, 25 cm × 4.6 mm I.D.; loading, 2 min at 5% acetonitrile in 0.1% aqueous TFA; gradient, step to 27% acetonitrile; 27 to 37% acetonitrile in (a) 15 min and (b) 150 min; flow-rate, (a) 1 ml/min and (b) 0.1 ml/min; sample, 30 mg of porcine insulin in 3 ml of 5% acetonitrile in 0.1% aqueous TFA.

Flow-rate (ml/min)	Purity (%)	Recovery (%)
1.0	99.95	63.0
	99.3	72.5
	98.7	80.4
	97.9	86.8
	96.7	91.9
	95.0	95.6
	93.2	97.9
0.1	100	88.8
	97.9	92.8
	93.7	96.9

dramatically. It is, therefore, important to operate the system at high efficiency if high recoveries are desired; this is often the case for recombinant proteins which typically have a very high value. High-efficiency operation usually translates to the use of low flow-rates because of the low diffusion rates of proteins. In such cases the production rate can be maximised by the use of small particles since at low flow-rates the operating pressure is usually low. With smaller particle diameters, the minimum in the Knox plot (of reduced plate height vs. reduced flow velocity) occurs at higher absolute velocities and the slope of the curve is generally less steep, allowing higher flow-rates.

The sample load in these experiments was 30 mg. The recovery and purity of a number of combinations of the fractions collected were calculated from both experiments. These results are shown in Table IV. The improved recovery of the run at 0.1 ml/min is seen clearly for the highest purity fractions. As the purity of the combined fractions is diminished,

there comes a point where the recovery from the experiment carried out with low flow-rate is less than that at faster flow. This is presumably because of the higher concentration of the impurity bands obtained at the lower flow-rate. The recovery from the combinations of fractions which yield products at 99.5% purity can be estimated to be 90% for the run at 0.1 ml/min and 70% for that at 1 ml/min flow-rate. This does not, of course, mean that the best flow-rate for the purification is 0.1 ml/min. The optimum flow-rate in preparative chromatography is a complex function of chromatographic parameters [purity, recovery, load and production rate (itself a function of flow-rate)], and economic factors (the cost of the starting material, the value of the product and the operating costs) [12]. Because the latter parameters are undefined in this example, the optimum flow-rate cannot be calculated.

It is interesting to compare these results for porcine insulin with those reported for the displacement chromatography of bovine insulin [4]. Because of the close similarity in structures, it is expected that there will be very little difference in the chromatographic behaviour of these two compounds and that comparison of the two experiments will be valid. Analysis of the displacement chromatographic data indicated that only one of the reported separations, carried out at a flow-rate of 0.1 ml/min with a load of 140 mg of bovine insulin on a column of dimensions 25 cm × 4.6 mm I.D., gave product pure enough for the comparison to be made. In this case, a purity of 98% was obtained with a recovery of 20%. Thus, in the 18.3 h of the experiment (110 ml at a flow-rate of 0.1 ml/min) a total of 28 mg at 98% purity could be collected. The purification of porcine insulin by gradient elution with the flow-rate of 0.1 ml/min was complete in 4 h and 10 min. In this time, a product of 98% purity could be isolated in 92.5% recovery, i.e. 27.8 mg. At the higher flow-rate (and a run time of 25 min), the recovery was less, but still 25.5 mg of porcine insulin at 98% purity could be isolated — a production rate 40 times higher than observed in the displacement separation. Because of the low recovery of high-purity material, it appears that the displacement separations reported may be more useful for an initial purification of crude material rather than a final preparation of high-purity product.

Effect of pore size

The pore diameter necessary for the effective chromatography of proteins has received some attention. For analytical separations, products with pore diameters of 300 Å are commonly available. More recently, it has been shown that the optimum pore diameter for preparative loading is smaller than this value, although this work was carried out statically and did not measure the dynamic effects [13].

The effect of pore diameter was studied using three packings of differing pore size. Two of these were spherical with pore diameters of 100 and 150 Å, respectively. The other was irregular, of 200 Å pore diameter. This last packing had a C_{18} bonded phase rather than the C_8 of the other phase. This was expected to reduce the effective pore size and surface area a little. The influence of pore size was studied by introduction of a given load upon the column and measurement of the change in retention time and the increase in band width over those observed for an analytical load. The theoretical plate height for a band is made up of contributions due to the band spreading from the column (small sample, *i.e.* kinetic effects) and the mass overload (thermodynamic effects) [14]. Since the band widths are proportional to the square roots of the individual plate height contributions, the mass overload band width is obtained by subtraction of the square of the analytical band width from that of the total band width and taking the square root of the result.

Both the change in retention at a given mass load and the mass overload band width are dependent upon the accessible surface area. This is a function of both the absolute surface area of the packing material and the pore diameter. Large molecules are excluded from the smaller pores of the packing and so only a certain percentage of the surface area is available for adsorption. When the surface area is high (and the pores therefore have small diameter), a relatively low percentage of the area is accessible. With increasing pore diameters, a greater percentage of the surface area is available, but the absolute area of the particle is reduced. Eventually, all of the surface is available as the pore becomes large compared with the molecules, but the surface area is reduced so much by the increase in pore size that very little solute can be adsorbed. Thus, for any molecular size there is an optimum combination of

pore size and surface area which allows a maximum loading.

The band width of a solute due to mass overload effects in gradient elution has been demonstrated to be approximately proportional to the square root of the mass load [13]. In order to ensure that the band widths measured in different columns would be related only to the different physical parameters of the particles within them, this relation was checked for porcine insulin. A graph plotting the logarithm of the mass-overload band width against the logarithm of the load was seen to be linear, with a slope of 0.53, very close to that reported earlier.

The changes in band widths and retention times due to mass overload caused by the injection of 2.5 mg of porcine insulin in the three columns are shown in Table V along with the experimental conditions. The minimum values occur for the 150-Å packing material, and this pore size is taken to be close to the optimum for this separation. In addition, the band width for an analytical load was found to be smallest for the 150-Å pore diameter packing. This is expected if the rate of mass transfer in and out of the pores is higher than for the 100-Å packing. The larger analytical band width for the 200-Å packing can be ascribed to its irregular particle shape; the effective particle size is probably larger than that of the spherical materials.

Effect of particle size

The effects of column efficiency upon the separation have been touched upon throughout this paper. Chromatography of the insulins was studied on

TABLE V

COMPARISON OF COLUMN PACKINGS UNDER 2.5 mg LOAD

Column, 25 cm × 4.6 mm I.D.; gradient, as Table IV, conditions a; sample, 2.5 mg porcine insulin in 250 μl of 5% acetonitrile in 0.1% aqueous TFA.

Packing material	Retention change (min)	Overload bandwidth (min)	Analytical bandwidth (min)
Kromasil 100	1.65	1.97	0.31
Kromasil 150	1.62	1.86	0.27
Poroquartz 200	1.66	1.89	0.39

columns with different particle sizes in order better to quantify the effects. It is known that for other protein separations the displacement effects are dependent upon particle size (i.e., column efficiency) [15]. For the example of cytochrome c and lysozyme, the maximum recovery was obtained only with a column efficiency above 1500 plates (for the protein). This was obtained in a 10-cm column packed with 15-μm particles. Since the selectivity for these proteins was high, it was expected that the minimum efficiency for the more difficult separation of insulins would be higher.

Columns 10 cm in length, packed with C_8 bonded-phase particles of 250 Å pore diameter and of 6.5, 20 and 50 μm, respectively, were used for the study. Sample loads of 10 mg of a 1:1 mixture of bovine and porcine insulins were used. The same gradient and flow-rate were used for all three columns, shown in Table VI. Fractions were collected through the peaks and were subsequently analysed. The purity and recoveries of combinations of fractions were calculated. These values are shown in Table VI. The recovery is markedly reduced as particle size is increased. With a particle diameter of 20 μm, the displacements are substantially degraded and for the 50-μm packing they are to all practical purposes eliminated. Extrapolation of the data suggests strongly that an acceptable recovery (85 to 90% at 99.5% purity) will be obtained only with particles of less than around 10 μm in the 10-cm columns used.

In order to obtain acceptable yields and purity it is clearly necessary to perform the separation with a high value of efficiency. This does not, of course, have to be achieved with particles as small as those used here; a column 20 to 50 cm long, packed with 13-μm particles would give almost the same separation as the 10-cm column packed with the 6.5-μm medium. Care must be taken if larger particles are used, because the large particle diameter means that the reduced plate height is also larger at constant flow-rate and either the flow-rate (and hence the production rate) must be decreased or disproportionately longer beds must be employed. When the separation is to be carried out at a large scale, the availability (and cost) of long columns has to be taken into account; in such cases, packing materials with small particle diameters become an essential part of the separation requirement [7].

TABLE VI

EFFECT OF PARTICLE SIZE ON PURITY AND RECOVERY

Column, Matrex 250 C_8, 10 cm × 4.6 mm, I.D.; gradient, as Table III, except that the step from 10 to 29% was made over a period of 10 min; other conditions as Table III.

Particle size (μm)	Bovine		Porcine	
	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)
6.5	100	95.9	100	55.6
			99.5	75.3
			99.0	84.7
20	100	61.3	100	12.3
	99.6	76.1	98.6	31.4
	98.7	87.3	96.9	56.2
	96.3	95.4	96.0	66.3
	89.7	98.6	89.3	84.4
			88.7	94.1
50	97.1	10.4	100	18.0
	94.8	37.1	96.0	31.7
	90.3	58.8	90.2	50.8
	86.0	73.5	88.0	58.3
	81.6	83.0		

CONCLUSIONS

Solute–solute displacements between the insulins were found under both gradient and isocratic conditions. Tag-along effects were not seen for the insulins, but were observed between an insulin and the corresponding desamido impurity. This implies that solute–solute displacements probably occur in the majority of preparative protein separations. The gradient slope was demonstrated to have relatively small effects upon the separation between the major components, but did affect the separation between the insulin and its desamido modification.

The influence of particle diameter upon the separation was profound. Reduction in column effi-

ciency by increase in the particle diameter of the packing eliminated the effects of the displacements. This had a major impact upon the purity and recovery of the components and demonstrated that high-efficiency operation is vital where solute–solute displacements are important to the purification.

The most important effect of flow-rate upon the chromatography of insulins was seen in the separation of the "tagged-along" desamidoinsulin, where a low flow rate — i.e. a high efficiency — improved the resolution between this and the main component.

The effects of pore diameter upon the loadability indicated that a pore diameter of approximately 150 Å was optimal for the preparative chromatography of insulins.

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